

Analytical Profiles of Drug Substances

Volume 11

Edited by

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PREFACE

It is now well over a decade that I perceived the need to supplement the official compendial standards of drug substances with a comprehensive review of pertinent physical, chemical, and analytical data and methods. Ten years ago the first volume of *Analytical Profiles of Drug Substances* was published under the auspices of the Pharmaceutical Analysis and Control Section of the APhA Academy of Pharmaceutical Sciences. That we were able to publish one volume per year is a tribute to the diligence of the editors to solicit monographs and even more so to the enthusiastic response of our authors, an international group associated with pharmaceutical firms, academic institutions, and compendial authorities. I would like to express my sincere gratitude to them for making this venture possible.

I am pleased to report that five years ago a companion series entitled *Pharmacological and Biochemical Properties of Drug Substances* was initiated by Morton E. Goldberg under the auspices of the section on Pharmacology and Toxicology, APhA Academy of Pharmaceutical Sciences. So far, three volumes have been published.

Over the years, we have had queries concerning our publication policy. Our goal is to cover all drug substances of medical value and, therefore, we have welcomed any monographs of interest to an individual contributor. We also have endeavored to solicit profiles of the most useful and used medicines, but many in this category still need to be profiled.

Starting with this, the eleventh volume, we shall also supplement previously published profiles with new data as we can find volunteers to write such supplements. In this volume, five of the original profiles in Volume 1 have been updated.

The goal to cover and update all drug substances of medical value with comprehensive monographs is still a distant one. I estimate that only about a quarter of such compounds have been profiled so far. We would very much like to accelerate

the rate of publication and hope that even more authors can be encouraged to write profiles. All those who have found these profiles useful are requested to contribute monographs of their own. We, the editors, stand ready to receive such contributions.

Klaus Florey

AMINOPHYLLINE

Kailas D. Thakker and Lee T. Grady

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1. Description

1.1 Nomenclature

1.11 Chemical Name

Aminophylline is chemically known as 1H-purine-2,6-dione,3,7-dihydro-1,3-dimethyl-, compound with 1,2-ethanediamine (2:1).¹

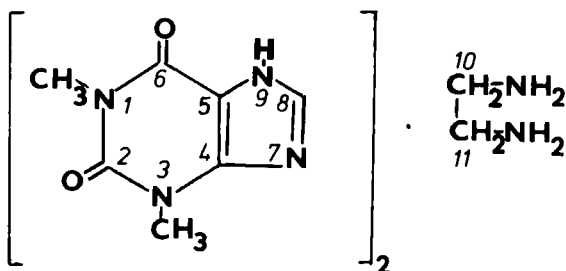
1.12 Adopted Names

Aminophylline was also known as theophylline ethylenediamine and euphylline.¹

1.13 Trade Names

Aminophylline is known as carena; inophylline; metaphylline; theophyldine; aminocardol; ammophylline; cardiocilina; cardophyllin; phylcardin; tefamin; cardiomin; grifomin; minaphil; peterphylline; stenovasan; theodrox; diophylline; genophylline; phyllindon and theolamine.¹

1.2 Formula, Molecular Weight, and Composition:



Anhydrous: $C_{16}H_{24}N_{10}O_4$ 420.44

Dihydrate: $C_{16}H_{28}N_{10}O_6$ 456.44

Theophylline 85-87%

Ethylenediamine 12-15%

1.3 Appearance, Color and Odor

Aminophylline is available in the anhydrous form or as the dihydrate. The dihydrate occurs as white or slightly yellowish granules or powder. It has a faint ammoniacal odor and a bitter taste.¹

2. Physical Properties

2.1 Spectral

2.11 Infrared

The infrared spectrum of aminophylline in mineral oil mull, obtained on a Beckman 4250 spectrophotometer, is shown in Figure 1.² It is generally consistent with the reported infrared spectrum of theophylline.^{3,4} The stretches for -NH_2 in ethylenediamine and -NH in theophylline appear as a broad band (combined with the mineral oil signal) in the region of 3.0-4.0 μM . Other signals are in the same vicinity as those for theophylline. The fingerprint region beyond 8.5 μM is distinctive and can be used for identification.

2.12 Ultraviolet

Spectral characteristics of aminophylline solutions in the ultraviolet region were reported by Andrade and Inacio.⁵ Absorption maxima occurred at 243-5 nm [$E_{1\text{cm}}^{1\%} = 170$] and at 273-5 [$E_{1\text{cm}}^{1\%} = 500$] in pH 9.5 borate buffer.

Figure 2 shows the ultraviolet spectrum of aminophylline in water obtained on a Beckman 5260 recording spectrophotometer.

2.13 Nuclear Magnetic Resonance

2.13.1 Proton NMR

An 80 MHz proton magnetic resonance spectrum of aminophylline in d_6 -dimethyl sulfoxide, obtained on a Varian FT-80A,⁶ containing tetramethylsilane as an internal reference, is shown in Figure 3. It is similar to the reported (60 MHz) proton NMR of theophylline.³ The assignments, based on assignments of theophylline protons, are shown in Table I.

2.13.2 Carbon-13 NMR

The 20 MHz proton-noise decoupled ^{13}C spectrum of aminophylline in d_6 -dimethyl sulfoxide, obtained on a Varian FT-80A is shown in Figure 4.⁶ The assignments are shown in Table II. These are based on assignments of dimethyluracil and 1-methylhypoxanthine.⁷

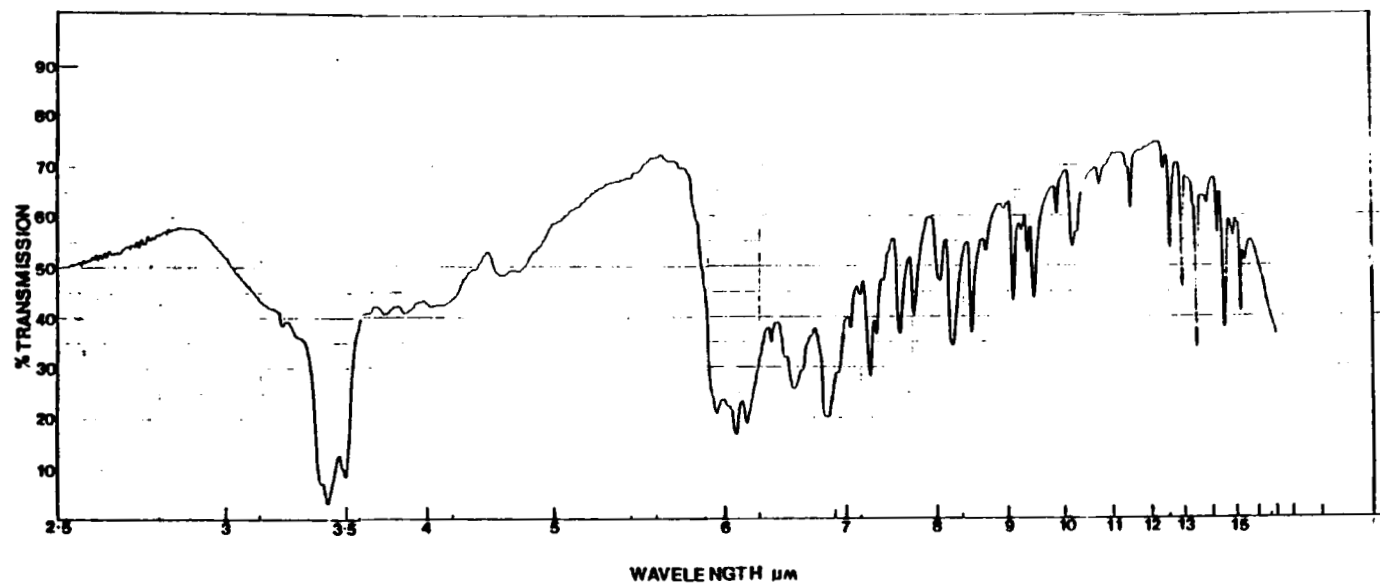


Fig. 1. IR Spectrum of Aminophylline

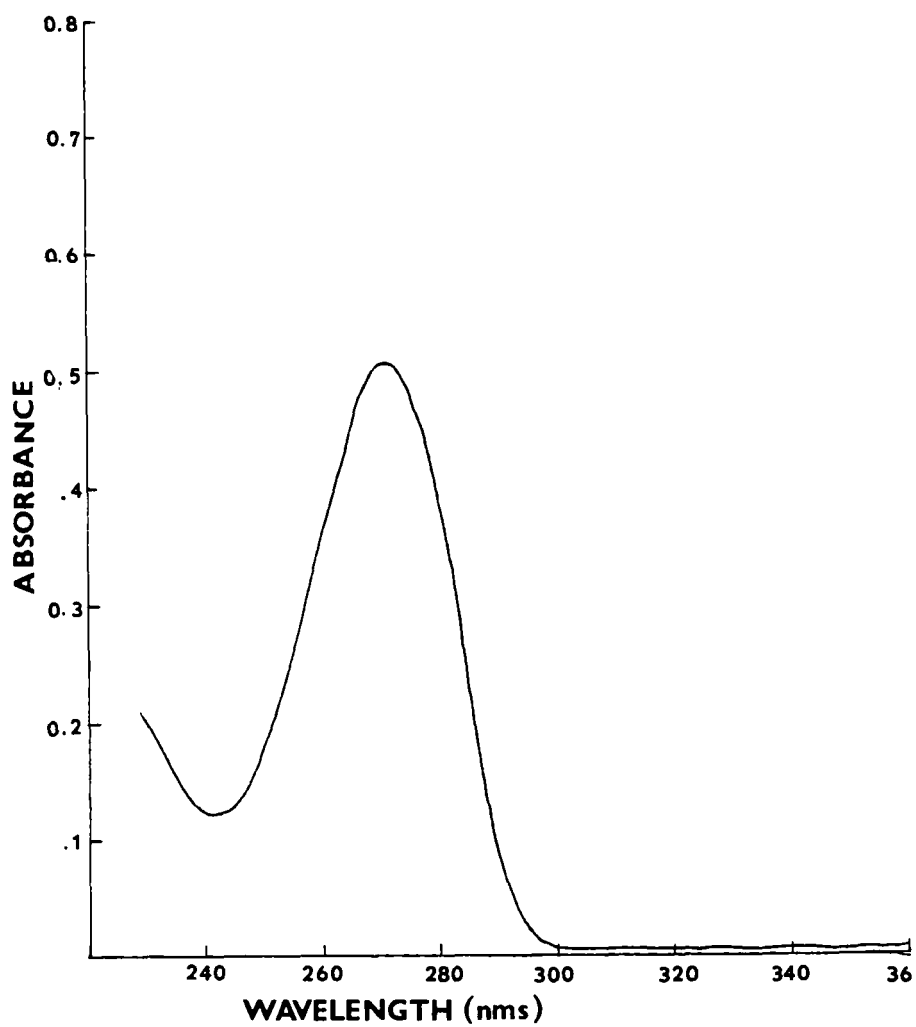


Fig. 2. UV Spectrum of Aminophylline

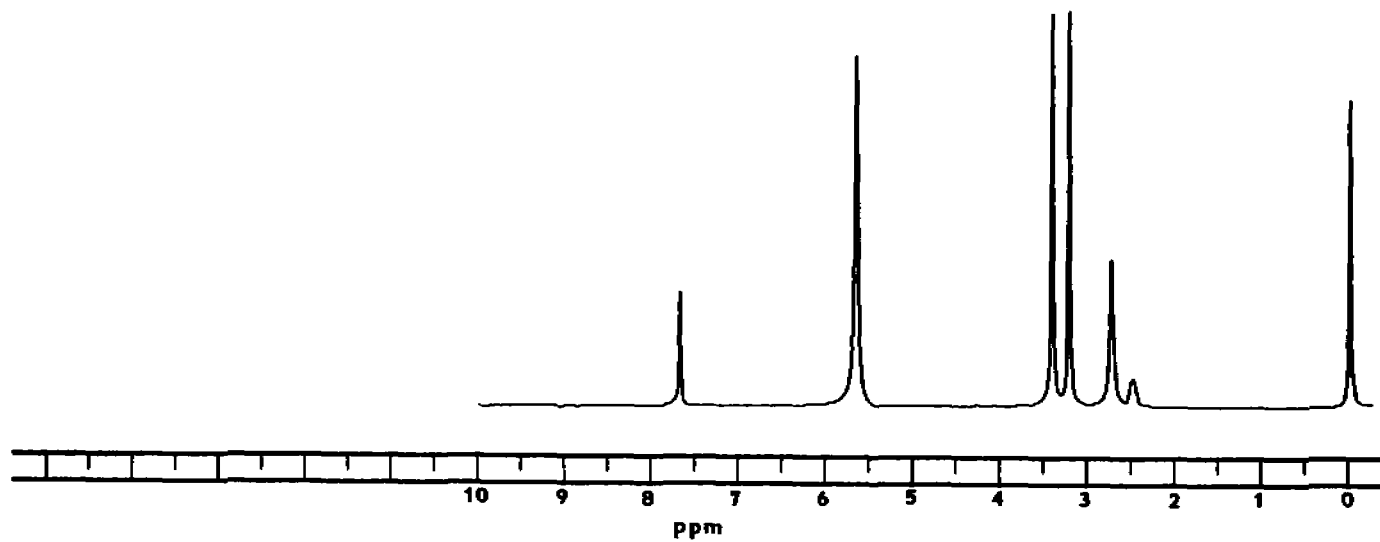


Fig. 3. ^1H NMR of Aminophylline

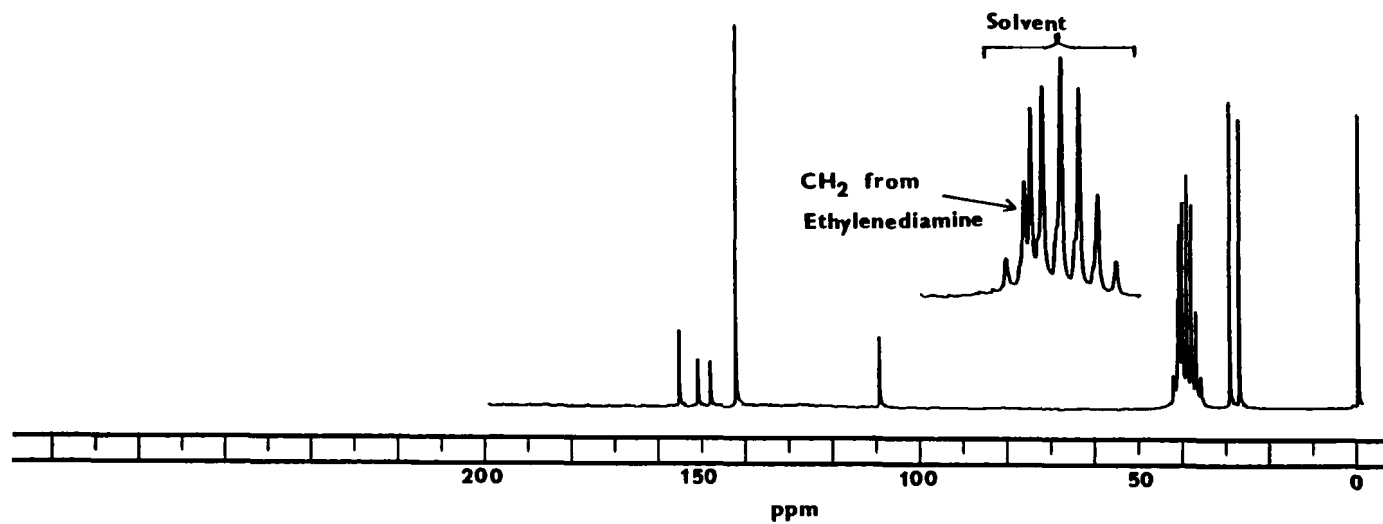


Fig. 4. ^{13}C NMR of Aminophylline

Table I

<u>Proton Assignment</u>	<u>Proton Position (see structure)</u>	<u>Chemical Shift (ppm)</u>
-CH ₂ -	10,11	2.75
-N-CH ₃ -	1	3.23
N-CH ₃ -	3	3.42
N-H ^a	7	5.67
C-H	8	7.71

^aIntensity of signal is proportional to the concentration.
This proton may be delocalized in the ring.

Table II

<u>Carbon Assignment</u>	<u>Carbon Position (see structure)</u>	<u>Chemical Shift</u>
N-CH ₃	1	29.7
N-CH ₃	3	27.5
C=C	5	109.6
C=C	4	148.5
N-C-N	8	142.7
-C=O	2	151.4
N-C=O-N	6	155.6

-CH₂ on ethylenediamine is buried in the solvent signal as shown in Figure 4.

2.2 Other Properties

2.21 Differential Scanning Calorimetry and Melting Point

The thermogram of aminophylline² shows two endothermic transitions, one at 120° and another at 272°C. The first transition reflects the melting point of aminophylline; the second transition reflects the melting point of theophylline. Theophylline is known to sublime on melting.²

2.22 Solubility

Aminophylline is soluble in water

(1 g/5 ml).¹ It is insoluble in dehydrated alcohol and in ether.⁸

3. Methods of Preparation:

Aminophylline was first prepared by Gruter⁹ by dissolving theophylline in aqueous solutions of ethylenediamine in stoichiometric proportions and evaporating in vacuum over sodium hydroxide. Alternate methods include treating anhydrous or hydrated crystals of theophylline with ethylenediamine vapor,¹⁰ and treating a 3 M solution of theophylline in a weak organic base (pyridine, quinoline or α -picoline) with a 2 M aqueous solution of ethylenediamine.¹¹

4. Stability-Degradation

4.1 Stability in Solution

Solutions of aminophylline become turbid on standing due to absorption of carbon dioxide, with subsequent precipitation of theophylline.^{1,8} During the preparation of aminophylline injection, excess ethylenediamine is necessary to keep aminophylline from decomposing.¹²

4.2 Stability in Solid State

Aminophylline crystals, in the presence of moisture, can absorb carbon dioxide from air and decompose into theophylline and ethylenediamine.⁸ This accounts for its characteristic odor.

Mixtures containing aminophylline and ephedrine hydrochloride were found to be discolored¹³ due to an exchange reaction between the two drugs. The ethylenediamine in aminophylline is presumed to liberate ephedrine base which decomposes rapidly. The color change was accelerated by temperature and humidity.¹³

Numerous reports are found in the literature on the stability of aminophylline in suppository bases,¹⁴⁻²¹ especially those containing fatty acids. Dissolution of suppositories made with cocoa butter base was markedly lower than those made with macrogol base after storage at 22° for up to a year.¹⁵ Other physical properties of cocoa butter suppositories such as melting point (Tm) and melting time have been known to increase within weeks of storage at 22°

and 30° as a result of decomposition¹⁶. Increase in the in vitro melting point (above 37° in some cases) was correlated with poor rate of release of theophylline.¹⁷

Decomposition of aminophylline suppositories is presumed to be due to the formation of insoluble amides between ethylenediamine and fatty acids of the suppository base.¹⁸ Four decomposition products that have been isolated¹⁸ were identified as mixtures of amides of oleic, palmitic, lauric and myristic acids.¹⁹ Further characterization of the decomposition products by IR, UV, GLC, and TLC confirmed the presence of alkyl amides.²⁰ Stabilizers such as hydroxylamine hydrochloride have been useful.²¹

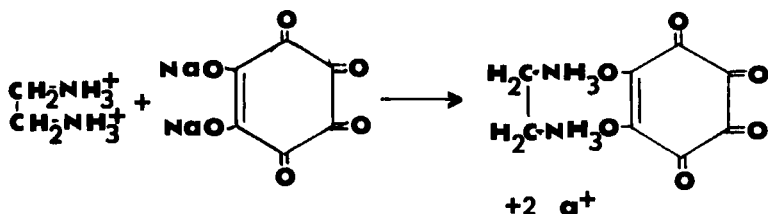
5. Methods of Analysis

5.1 Identification Tests

Spectral identification tests include IR, UV, Mass and NMR spectroscopy. The melting point of theophylline liberated from aminophylline is the basis of one of the compendial identification tests.²²

The following color reactions are also useful identification tests; many are used for dosage forms and for mixtures of pharmaceuticals.

(i) Ethylenediamine present in aminophylline reacts with sodium rhodizonate to form a water-soluble, violet-colored precipitate.²³ Ethylenediamine must be released from aminophylline and converted to acetate or hydrochloride.



(ii) Ethylenediamine also forms a yellow precipitate with 2,4-dinitrochlorobenzene.²⁴ Primary aliphatic and aromatic amines interfere with the test.

(iii) Aminophylline develops an orange color with ferric chloride and a yellow color with Ehrlich's reagent. Color development is rapid. This test was designed by Cooper²⁵ for identification of pharmaceuticals in tablets.

(iv) Reaction between dimethylglyoxime and oxidized solutions of purines to form a colored product is the basis of the identification test developed by Kido.²⁶

(v) Reaction of theophylline with potassium chlorate in hydrochloric acid, followed by exposure to ammonia vapors, produces a purple residue. This reaction is the basis of one of the compendial identification tests for aminophylline.²²

(vi) Bratton-Marshall: Aminophylline in acid solution, when treated with diazotized p-aminobenzene-sulfonic acid or p-aminonitrobenzene produces a yellow or a red color, respectively.²⁷

(vii) Addition of 1-2 drops of a 5% solution of sodium nitroprusside to a solution of aminophylline in acetone produces a violet color.²⁸

(viii) Aminophylline powder, on mixing and heating with copper sulfate powder, also produces a violet color.²⁹

5.2 Gravimetric Methods

Earlier methods of analysis of aminophylline were based on extraction of theophylline with organic solvents such as chloroform and 2-propanol, followed by gravimetric determination of the residue.^{30,31}

5.3 Titrimetric Methods

5.31 Alkalimetric

The weakly acidic nature of theophylline lends itself to titrations with alkali. Titrations with alkali have been carried out to assay aminophylline using a potentiometric³² or colorimetric³³ end-point (thymolphthalein as indicator) in mixtures of pharmaceuticals.

5.32 Acidimetric

Ethylenediamine's basic nature was used in many early methods of analysis in which titrations with strong acid were carried out.^{31,34,35} Separation of ethylenediamine by solvent extraction³¹ or ion-exchange chromatography³⁴ was followed by titrations with hydrochloric acid using a colorimetric end-point (methyl orange as indicator).

5.33 Argentometric

When theophylline is treated with solutions of silver nitrate, it forms a silver salt that is insoluble in water and soluble in nitric acid solutions. Based on this property, several assay methods have been developed,³⁶⁻⁴¹ including the compendial assay for aminophylline.²² In general, silver theophyllate is precipitated, filtered, washed, re-dissolved in nitric acid and titrated with ammonium thiocyanate. Aminophylline present in mixtures of pharmaceuticals^{36,37,39} and xanthines³⁸ can be analyzed. Some presence of ammonia is necessary when precipitating silver theophyllate.⁴⁰ It has been suggested that argentometric titrations give higher values due to adsorption of silver ions by the very voluminous silver theophyllate precipitate.³¹

In an unusual application, silver (¹¹⁰Ag) nitrate was used and radiometric titration was carried out to analyze aminophylline.⁴¹

5.34 Complexometric

Bosly developed a titrimetric method for aminophylline by precipitating Hg-(theophylline)₂ with mercuric acetate, and titrating excess Hg⁺⁺ ions with ammonium thiocyanate.⁴² Similarly, the Cu salt of theophylline is also precipitated and excess copper titrated with ethylenediaminetetraacetate.^{43,44}

5.35 Non-Aqueous

Non-aqueous titrations of aminophylline using sodium methoxide as the titrant have been carried out with dimethylformamide⁴⁵ or benzene-methanol³³ as the solvent for aminophylline. A differential titrimetric method for aminophylline was developed using acetous perchloric acid as the titrant.⁴⁶ This method allows

determination of ethylenediamine and theophylline content in a single titration, and is useful for aminophylline tablets, injections and suppositories.

5.4 Spectroscopic Methods

Aminophylline solutions obey the Beer-Lambert Law for a concentration range of 0.5-1.2 mg%.⁵ Schack and Waxler⁴⁷ developed a potentiometric assay for aminophylline in biological fluids using chloroform/2-propanol extraction to isolate theophylline, followed by UV-absorbance measurement at 275 nm. The method of Schack and Waxler was used most extensively for analysis of aminophylline in early pharmacokinetics research.^a Several other spectrophotometric methods have been developed for aminophylline in mixtures of pharmaceuticals, each one using an extraction with organic solvent to isolate aminophylline.^{48,49} In one case, graphical correction was applied to the UV absorbances of mixtures of aminophylline and phenobarbital, measured at two wavelengths of maxima.⁵⁰ In another application, extraction of serum samples with a salt-solvent pair of ammonium sulfate and chloroform:hexane (7:3 v/v) was carried out followed by back extraction of theophylline^a into aqueous borate buffer (pH 9.0) and measurement of UV absorbance at 275 nm.⁵¹ Plavsic⁵² used charcoal extraction to isolate theophylline from other interfering substances. Since theophylline was reversibly adsorbed on charcoal, elution with organic solvent was followed by measurement of UV absorbance.

Determination of aminophylline in the blood of patients was carried out after oxidation with potassium dichromate in an acidic medium,⁵³ separation of the oxidation product by steam distillation and measurement of UV absorbance at 257 nm.

5.5 Chromatographic Methods

5.51 Thin-Layer Chromatography

In addition to systems developed for theophylline,³ several TLC systems have been developed for

^aAminophylline and theophylline are indistinguishable at biological pH's. Therefore assays of theophylline in biological fluids are also included here due to their obvious applicability.

Table III

Thin-Layer Chromatographic Systems for Aminophylline

No.	Solvent for Drug	Stationary Phase	Developing Solvent	Detection Methods	Application	Ref.
1.	chloroform	Silica gel F ₂₅₄	chloroform:acetic acid (100:20)	UV, Drogen- dorff's reagent	Analysis of suppositories	54
2.	water	aluminum oxide	benzene:ethanol (9:1 or 9:1.5)	UV, iodine, modified Dragendorff's reagent	Mixture of pharmaceuticals	55
3.	----- ^a	Silica gel F ₂₅₄	(i) acetone:chloro- form:1-butanol:25% ammonium hydroxide (30:30:40:10) (ii) chloroform:ethyl ether (90:10) (iii) chloroform:eth- anol (90:10)	ferric chloride, iodine	Analysis of mixtures of xanthines	56
4.	----- ^a	Silica gel H	chloroform:acetone: methanol (8:1:1)	UV	Analysis of suppositories	18

Table III (contd.)

No.	Solvent for Drug	Stationary Phase	Developing Solvent	Detection Methods	Application	Ref.
5.	----- ^a	Silica gel F ₂₅₄	methylene chloride: methanol:acetic acid (90:10:3)	UV	Analysis of tab- lets ampuls, suppositories	57

^anot mentioned.

analysis of aminophylline and its dosage forms. Table III lists the methods developed for aminophylline.

Zorka et al⁵⁸ separated and identified several pharmaceuticals in a mixture including aminophylline using silica gel G plates and neutral, acidic, and alkaline mobile phases. Detection was done by UV.

Riechert⁵⁹ developed a "micro"-TLC method for analysis of theophylline in biological fluids. Kieselgel 60 F-254 DL-"Fertigplatten" thin-layer plates were used and developed with chloroform:methanol (90:10) for a mixture of caffeine and theophylline and with ethyl acetate:methanol:25% ammonia (80:20:10) for a mixture of theobromine and theophylline present in saliva, plasma or urine. Sensitivity of detection claimed was 25 ng/10 μ l. None of the dietary xanthines or other commonly co-administered drugs appear to interfere.

5.52 Paper Electrophoresis⁶⁰

Whatman Paper No. 1 was used with a potential gradient of 20 V/cm. Several xanthines were separated. Among these, theophylline and aminophylline were best chromatographed in Britton-Robinson buffer at pH 10. Detection agent was 1% solution of disodium-2-hydroxy-3,6-naphthalenedisulfonate.

5.53 Pressurized Liquid Chromatography

Numerous liquid chromatographic methods for determinations of theophylline in biological fluids are listed in the literature.^{3,61-92} The majority of the methods use reverse-phase chromatography,⁶¹⁻⁸² few are listed that use either normal-phase^{83,84} or ion-exchange chromatography.^{85,86} In many cases, chromatographic conditions have been developed for a specific application,^{87,88,89} since adaptation of reported methods may introduce some interference from co-administered drugs such as acetazolamide,⁹⁰ trisulfapyrimidine⁹¹ or cephalosporins.⁹²

Table IV lists some of the recent, most cited methods of analysis of theophylline in biological fluids by liquid chromatography.

Table IV

No.	Stationary phase	Mobile Phase	Pre-treatment of sample	Comments	Ref. No.
1.	Reverse-phase C-18 (ion-pair)	methanol:sodium acetate (pH 4.2) containing 10 mM tetrabutylammonium chloride (10:90)	Extraction of plasma with chloroform:2-propanol (50:50)	Separation of theophylline from paraxanthine.	61
2.	Reverse-phase C-18 (ion-pair)	acetonitrile:water containing 10 mM tetrabutylammonium chloride (2.5:97.5)	Urine: pH adjusted before chromatography Serum: ultrafiltration	Analysis of theophylline in urine and serum; separation from other xanthines and metabolites.	62
3.	Reverse-phase C-18	ethanol:water (20:80)		Assay of theophylline in plasma; separation of theophylline from sulfisoxazole and ampicillin.	63
4.	Reverse-phase C-18 (μ -Bondapak C-18)	acetonitrile:0.01 M sodium acetate (pH 4.0) (1:9); 2.0 ml/min flow	Molecular ultrafiltration to remove plasma proteins	Direct injection.	64

Table IV (contd.)

No.	Stationary phase	Mobile Phase	Pre-treatment of sample	Comments	Ref. No.
5.	Reverse-phase C-18	acetonitrile:water (6:94); 3.0 ml/min flow	De-proteinization with 2.5 volumes of acetonitrile	Micromethod--only 10- μ l sample is required.	65
6.	Reverse-phase C-18 (μ -Bondapak C-18)	acetonitrile:acetate buffer (pH 4.0) (7:93)	De-proteinization with aqueous acetonitrile-solution	Microscale method--only 30 μ l plasma needed for analysis; direct injection.	66
7.	Reverse-phase C-18 μ -Bondapak C-18	methanol:1% propionic acid (20:80)	Extraction with chloroform evaporation; sample redissolved in methanol	50- μ l sample needed.	67
8.	Reverse-phase C-18 (μ -Bondapak C-18)	methanol:tetrahydrofuran:water containing 10 mM/liter sodium acetate (40:10:50)	Same as Ref. 62	<u>p</u> -Hydroxyethyltheophylline as internal standard; separation from other xanthines and commonly administered antibiotics.	68

Table IV (contd.)

No.	Stationary phase	Mobile Phase	Pre-treatment of sample	Comments	Ref. No.
9.	Reverse-phase	methanol:sodium acetate (15:85)	Deproteinization of serum samples with two volumes of methanol	β -Hydroxyethyltheophylline used as internal standard; no interference from antibiotics or metabolites.	69
10.	Reverse-phase	methanol:ammonium phosphate	Extraction with organic solvent before analysis	100 μ l of serum sample can be analyzed; theophylline can be analyzed in presence of anticonvulsants.	70
11.	Reverse-phase C-18	acetonitrile: acetate buffer (pH 4.0) (90:10)	Extraction with chloroform:2-propanol (95:5)	β -Hydroxyethyltheophylline used as internal standard; theophylline recovery was found to be between 71 and 75%.	71

Table IV (contd.)

No.	Stationary phase	Mobile Phase	Pre-treatment of sample	Comments	Ref. No.
12.	Reverse-phase C-18	acetonitrile: acetate buffer (pH 4.0) (12:88)	Extraction of 0.5-0.2 ml of acidified plasma with dichloromethane	Separation of xanthines; sensitivity of detection 0.1 mg/liter	72
13.	Reverse-phase C-18	methanol:0.05 M phosphate buffer (pH 4.7) (12:88 v/v)	Molecular filtration of serum to separate proteins	Method applicable to human serum, urine and saliva samples, also separation and quantitation of theophylline and its metabolites.	73
14.	Reverse-phase			20 µl of blood are sufficient for analysis; detection by direct-current pulse and differential pulse amperometry.	74

Table IV (contd.)

No.	Stationary phase	Mobile Phase	Pre-treatment of sample	Comments	Ref. No.
15.	Reverse-phase C-8	methanol:buffer (14:86 v/v)	Extraction with 2-propanol:chloroform (25:75)	Completely automated analysis. Developed on Technicon "Fast-LC". 200- μ l sample required. No interference was observed.	75
16.	Reverse-phase RP-8	sodium acetate: 0.02 M methanol (2:1)	Adjustment of pH to 5.5	Theophylline, sulfamethoxazole, ampicillin and caffeine well separated.	76
17.	Reverse-phase C-18	acetonitrile: phosphate buffer (pH 4.8) (1:1); 3.0 ml/min flow; 50°	Same as Ref. #5	Authors found that the retention time of internal standard varied from run to run when using the conditions described in Ref. #5; column temp. was therefore maintained at 50°C.	77

Table IV (contd.)

No.	Stationary phase	Mobile Phase	Pre-treatment of sample	Comments	Ref. No.
18.	Reverse-phase C-18 Whatman Partisil 10-ODS	acetonitrile: 10 mM phosphate buffer (10:90)	Protein denaturation by acetonitrile	No pre-column nec- essary; no inter- ference observed.	78
19.	Reverse-phase C-18 μ -Bondapak	methanol:pH 2.0 hydrochloric acid solution (containing 0.02 M potassium chloride)	Precipitation of proteins by 50% v/v trichloroac- etic acid	Theophylline ana- lyzed in presence of methyl xanthines and caffeine.	79
20.	Reverse-phase C-18	acetonitrile:ace- tate buffer (8:92)	Plasma samples extracted with chloroform:2-propanol (95:5); solvent removed and samples re-dissolved dissolved in mobile phase	Theophylline well separated from paracetamol and and xanthines.	80
21.	Reverse-phase C-18 5 μ	Solvent A: water containing .01 M sodium acetate and 0.005 M te- trabutylammonium hydrogen sulfate.	Ion-pair extraction using tetrabutylammonium sul- fate and ethyl acetate: chloroform:2-propanol, (45:45:10 v/v) after ad- justment of urine pH to 6.0-6.5	Simultaneous quan- titation of theo- phylline and its major metabolites.	81

Table IV (contd.)

No.	Stationary phase	Mobile Phase	Pre-treatment of sample	Comments	Ref. No.
21. contd.		Solvent B: (50:50) methanol:solvent A gradient elution with 9% solvent B at start, 46% solvent B at end of run (for program see Ref. 81).			
22.	Reverse phase μ -Bondapak C-18	methanol:10 mM monobasic sodium phosphate (1:4); 0.8 ml/min flow	Proteins precipitated by perchloric acid, supernatant neutralized and injected	50 μ l of serum are sufficient; theophy- lline is well separ- ated from dietary xanthines, caffeine, theobromine and theophylline meta- bolites.	82

Table IV (contd.)

No.	Stationary phase	Mobile Phase	Pre-treatment of sample	Comments	Ref. No.
23.	Normal phase column (silica)	chlorform:dioxane: formic acid (95.5:4.5:0.01 v/v)	Equal volume of saturated ammonium sulfate is added to plasma, and then extracted with chloroform:2-propanol (95:5 v/v); solvent is evaporated and residue redissolved in mobile phase	100 µl of plasma are sufficient; theophylline well separated from the metabolites.	83
24.	Normal phase Lichrosorb Si-60	(i) chloroform: 2-propanol: glacial acetic acid (92:7:1) with 40% hexane (ii) methylene chloride: methanolic ammonium formate (98:2)	Extraction from plasma with chloroform 2-propanol (95:5)	Mass spectrometry used for identification.	84
25.	Cation-exchange Partisil SCx column temp. at 50°C	0.66% acetic acid	0.1-ml sample extracted with ethyl acetate	Applicable to plasma or saliva samples; no interference.	85

Table IV (contd.)

No.	Stationary phase	Mobile Phase	Pre-treatment of sample	Comments	Ref. No.
26.	Strongly basic anion ex- change resin (35% cross- linkage)	acetate buffer; linear gradient from 0-6.0 M, flow 0.72 ml/min	Sample filtered to remove particulate matter	General method for UV-absorbing com- pounds in urine; ≈ 30 compounds tested.	86

5.54 Gas Chromatography

Earlier attempts to analyze blood levels of theophylline resulted in the development of various gas chromatographic methods.⁹³⁻¹⁰² Most of these methods require extraction and derivatization before chromatography. Table V lists some of the more recent methods.

5.6 Immunoassays

5.61 Enzyme Immunoassay (EMIT)

The enzyme immunoassay (EMIT) developed by Syva Corporation (Palo Alto, California) is the most widely used method for the assay of theophylline (in biological fluids) in clinical laboratories.¹⁰³

In principle, theophylline antibodies are prepared¹⁰⁴ by injecting a solution of bovine gamma globulin linked to theophylline (or a chemically similar derivative) into sheep. Theophylline (or a derivative) is also linked to an enzyme, in this case, glucose-6-phosphate dehydrogenase. When a patient's serum containing free theophylline is mixed with a solution of antibodies and enzyme-labelled theophylline, the free drug and the enzyme-labelled drug compete for the binding sites on antibodies. The reduction in enzyme activity when bound to antibodies can be monitored by using the proper substrate; in this case NADH. This assay is rapid, specific, and requires small sample size.¹⁰⁵⁻¹⁰⁷ It is easily adaptable to commercial kinetic analyzers,¹⁰⁸⁻¹¹⁷ and can be modified¹¹⁸ to suit the application. Comparison of the EMIT assay with chromatographic methods shows good agreement between the two assays.^{106,119}

Vinet et al¹²⁰ developed another enzyme immunoassay. In this method, the sample is extracted with chloroform/2-propanol, and back-extracted into aqueous sodium hydroxide. Inhibition of beef liver phosphatase by theophylline is determined at 25° C, using *p*-nitrophenyl acetate as the substrate in a pH 9.4 2-amino-2-methyl-1-propanol buffer system.

In another application, nephelometric, competitive immunoassay was developed.¹²¹ A precipitate was obtained by combining theophylline-antibody complex with a macromolecule, and scattering of light by the precipitate

Table V

No.	Stationary phase	Conditions	Pre-treatment of sample	Comments	Ref. No.
1.	3% OV-17 on Gas Chrom Q	Column at 230°C; nitrogen-phosphorus detector	Sample extracted with chloroform:2-propanol (50:50), evaporation and redissolution of residue in 0.02 M tetrabutylammonium hydroxide	20 µl of plasma are sufficient; detection sensitivity 100 µmol/liter; theophylline well separated from other xanthines and co-administered drugs.	93
2.	Silicone stationary phase, 2% SP 2510 DA	F.I.D.	Extraction of sample with salt-solvent pair of ammonium sulfate and methylene chloride: hexane:acetic acid (80:20:0.1)		94
3.	5% OV-225 on 80/100 Gas Chrom Q	Electron-capture detector; column at 250°C	Sample extracted with ethyl acetate, and then treated with pentafluorobenzyl bromide for derivatization	100 µl of serum are sufficient; detection sensitivity 0.1 µg/ml.	95

Table V (contd.)

No.	Stationary phase	Conditions	Pre-treatment of sample	Comments	Ref. No.
4.	3% XE-60 on 80/100 Gas Chrom Q	Electron-capture detector; injector and column at 220°C, detector at 225°C	Derivatization by pentafluorobenzyl bromide followed by column chromatography prior to injection	Sensitivity of detection 5 ng/ml.	96
5.	3% OV-17 on 100/120 Gas Chrom Q	Nitrogen detector; column at 240°C	Extraction with tetrahexylammonium hydrogen sulfate in aqueous sodium hydroxide	25-μl sample is sufficient.	97
6.	3% OV-17 on 100/120 Gas Chrom Q	Nitrogen-phosphorus detector; column at 240°C	Off-column derivatization as follows: Sample is extracted with dichloromethane, dried and reacted with N,N-dimethylacetamide, tetramethylammonium hydroxide and 1-iodopentane. It is then transferred into cyclohexane: pentane mixture (95:5), the solvent evaporated, and it is redissolved in methanol.	No interference from drugs or metabolites.	98

Table V

No.	Stationary phase	Conditions	Pre-treatment of sample	Comments	Ref. No.
7.	3% OV-17 on Gas Chrom Q	Flame-ionization detector; column at 190°C	Extraction of sample with ether:dichloromethane: 2-propanol (6:4:1), re-extraction of the organic layer with 1 N sodium hydroxide, acidification with phosphoric acid, re-extraction with organic solvent, evaporation and redissolution of residue in tetrapropylammonium hydroxide		99
8.	3% SP 2250 on 100/120 Supelcoport	Flame-ionization detector; column temperature programmed from 160°C to 240°C at 8° C/min	Sample extracted with dichloromethane, evaporated, and dissolved in toluene, butylating agent is then added.		100

Table V (contd.)

No.	Stationary phase	Conditions	Pre-treatment of sample	Comments	Ref. No.
9.	3% OV-17 on 100/120 Chrom W HP column well conditioned	Column temperature programmed from 180°C to 280°C; detector at 280°C, mass spectral source at 150°C	Extraction with chloroform, butylation with tetramethylammonium hydroxide and <u>N,N</u> -dimethylacetamide	Mass spectrometry using probability-based matching.	101
10.	3% SP2250 on 100/120 Supelcoport type 50-50 methylphenyl silicone	Column temperature programmed at 190°C to 300°C at 10°/min	Samples are introduced in a flash heater for ethylation	No interference.	102

was used to quantitate theophylline content.

5.62 Radioimmunoassay (RIA)

The principle of radioimmunoassay is similar to EMIT, except that in this case decrease in radioactivity is measured. Radioimmunoassays for theophylline have been developed using ^3H -theophylline.^{122,123} 8-Carboxy-theophylline was used to prepare antibodies. There is no interference from endogenous purines or known metabolites of theophylline at the concentrations studied.¹²²

6. Metabolism

Using [^{14}C] aminophylline injection, Caldwell, Monks and Smith¹²⁴ determined that the metabolites of aminophylline are the same as those of theophylline.^{125,126} These are (i) 3-methylxanthine (ii) 1,3-dimethyluric acid and (iii) 1-methyluric acid. However, the rate and extent of conversion to 1,3-dimethyluric acid and 3-methylxanthine were higher for aminophylline than for theophylline.¹²⁶ Therefore, ^{14}C recovery in urine (0-24 hours) was higher for aminophylline (87%) than for theophylline (76%). The formation of 3-methylxanthine follows saturation kinetics; therefore, the presence of circulating methylxanthines from foods affects the elimination of aminophylline.¹²⁷ The appearance of the other two metabolites follows first order kinetics. Jenne et al.¹²⁸ determined that 1-demethylation of theophylline to 3-methylxanthine is the dominant reaction determining theophylline concentration in serum. Presence of ethylenediamine must affect this conversion, but how it does is not known.¹²⁷

7. Biopharmaceutics and Pharmacokinetics

Pharmacokinetics of aminophylline has been studied extensively. Since aminophylline and theophylline are indistinguishable in biological fluids, pharmacokineticists do not differentiate between the two. Although the pharmacokinetics of theophylline was reported³ earlier, the advent of newer analytical techniques has since led to an extensive amount of work.

Aminophylline is administered orally,¹²⁹⁻¹³³ as a sustained-release dosage form^{132,133,134,135} or as a single-dose capsule¹³⁶ or tablet,^{134,130} intravenously (or by infusion),¹³⁷ intramuscularly, or rectally as suppositories or enema.^{138,131} Among different aminophylline dosage

forms, rectal suppositories give the widest variation.^{138,131}

Absorption of aminophylline from tablets or capsules is rapid, plasma levels reaching therapeutic range within 1-1 1/2 hours. Sustained-release preparations of aminophylline are used often to maintain theophylline levels within therapeutic range for about 12 hours in treatment of asthma.^{132-135,139} The rectal route is used often with infants and children.

Plasma theophylline levels of 5-15 µg/ml (after administration of aminophylline) are considered safe and therapeutic.¹⁴⁰ Although in most cases toxic symptoms do not appear at levels above 25 µg/ml,¹⁴⁰ the patient-to-patient variation is so high that individualization of the therapy is necessary.¹⁴¹⁻¹⁴⁵ In some cases, intra-patient variation in dose/blood levels is observed¹⁴⁶ during continuous long-term therapy. Saliva and breast milk levels of theophylline are lower than plasma levels,¹⁴⁷ but a good correlation between plasma/saliva ratio is obtained.¹⁴⁷⁻¹⁴⁹

The pharmacokinetic behavior of aminophylline once it appears in the blood can be described by the same model that is used to describe theophylline pharmacokinetics.³ The volume of distribution at steady-state for all ages is ≈ 0.45 liter/kg,¹⁵⁰⁻¹⁵¹ except in neonates where it is slightly larger.¹⁵² Elimination is rapid, with a half-life of about 6 hours in normal, healthy, non-smoking adults. Among the factors that affect the elimination of theophylline from plasma are age,¹⁵³⁻¹⁵⁷ physiology and disease state,¹⁵⁸⁻¹⁶² co-administered drugs,¹⁶³⁻¹⁶⁴ diet,¹⁶⁵ time of day,¹⁶⁶ and smoking habits.¹⁶⁷⁻¹⁶⁹ In a case when any of these factors is operating, the half-life of elimination varies from 5 to 30 hours.

Very recently, Monks et al¹²⁷ have compared the disposition and elimination of theophylline and aminophylline. Elimination of aminophylline was faster than theophylline in the same subjects. Although qualitatively the disposition (and metabolism) of theophylline and aminophylline were similar, the authors claimed there were small but significant differences in rates of elimination and the extent of dose eliminated within 48 hours.

8. Toxicity

Toxicity of aminophylline results when blood levels exceed the level of 20 $\mu\text{g/ml}$ of theophylline.¹⁷⁰ The severity of toxicity is directly related to plasma levels of theophylline. In mild toxicity, symptoms are nausea and vomiting,¹⁷⁰⁻¹⁷¹ diarrhea, abdominal pain, nervousness, insomnia, tachycardia and headache. In severe toxicity, serious tachycardia, grand mal seizures,^{170,171-173} and cardiac arrhythmias may occur. In some cases, death may result from acute toxicity.¹⁷⁴

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ASCORBIC ACID

Ibrahim A. Al-Meshal and Mahmoud M. A. Hassan

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1, Description

1.1. Nomenclature

1.1.1 Chemical Names

- a) L-Ascorbic acid
- b) L-Xyloascorbic acid
- c) 3-Oxo-L-gluofuranolactone (enol form).
- d) L-3-Ketothreohexuronic acid lactone

1.1.2 Generic Names

Vitamin C; Ascorbic acid

1.1.3 Trade Names

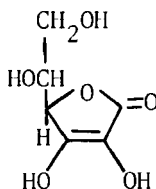
Adenex; Allercorb; Antiscorbutic Vitamin; Ascorbicap; Ascorbajen; Ascoril; Ascorin; Ascorreal; Ascorvit; Cantan; Cantaxin; Catavin C; Cebicure; Cebid; Cebione; Cecon; Cegiolan; Cellin; Cenetone; Cereon; Cergona; Cescorbat; Cetamid; Cetan; Cetemican; Cevalin; Cevatine; Cevex; Cevimin; Cevi-Bid; Ce-Vi-Sol; Cevitamin; Cevitan; Cimin; Cevitamie Acid; Cevitex; Ciamin; Cipca; Colascor; Concemin; C-vimin; Davitamon C; Erivit C; Hybrin; Laroscorbine; Lemascorb; Megascorb; Planavit C; Proscorbin C; Redoxon; Ribena; Scorbacid; Scorbu-C; Testascorbic; Vicelat; Vitace; Vitacimin; Vitacin; Vitascorbol; Vitix.

1.2 Formulae

1.2.1 Empirical



1.2.2 Structural



1.2.3 CAS No.

(50-81-7)

1.2.4 (Wiswesser Line Notation)

TOSV EHJ CQ DQ EYQ IQL (1)

1.2.5 Stereochemistry

The nature of the ring system in ascorbic acid was determined by a study of the methylated derivatives of the acid. By this means complete confirmation was obtained of the accuracy of the views advanced, concerning the stereochemical configuration of the molecule and the nature of the reactive enolic groups(2). The furanose structure for ascorbic acid shown above (1.2.2) was put forward by Lerbert *et al* (2) on the basis of its chemical behaviour as well as its oxidation products.

1.3 Molecular Weight

176.12

1.4 Elemental Composition

C, 40.91%; H, 4.58%; O, 54.51%.

1.5 Appearance, Color, Odor and Taste

White or slightly yellow crystals or powder. Odorless or almost odorless; pleasant sharp acidic taste(3).

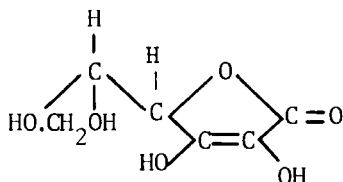
2. Physical Properties2.1 Crystal Properties

Usually plates, sometimes needles, monoclinic system (4).

2.1.1 X-ray Diffraction

The available data (2) of the X-ray, reveals that the total of 12 carbon and oxygen atoms

all but one can be accommodated in one plane without appreciable valency strain whilst the remaining carbon (C₅) lies less than 1A° above the plane as shown in the following model:



2.1.2 Melting Range

Ascorbic acid melts at 190-192° with decomposition (4).

2.2 Solubility

Ascorbic acid is soluble at 20°, in 3.5 parts of water and 25 parts of alcohol (95 per cent); 50 parts of absolute alcohol, 100 ml of glycerol, 20 ml of propylene glycol. Solubility in hot water 40.0% at 40°, 80% at 100°. Insoluble in ether, chloroform, benzene and light petroleum (boiling range 40-60°).

2.3 Specific Rotation

$$[\alpha]_D^{25} + 20.5^\circ \text{ to } 21.5^\circ \text{ (C = 1, water)}$$

$$[\alpha]_D^{23} + 48 \text{ (C = 1, methanol) (4).}$$

$$[\alpha]_{5780}^{19} + 24 \text{ (water) (2).}$$

$$[\alpha]_{5780}^{18} + 116 \text{ (sodium salt in neutral solution) (4)}$$

$$[\alpha]_{5780}^{18} + 130 \text{ (N/20 NaOH) (2).}$$

$$[\alpha]_{5780}^{18} + 149 \text{ (N/1 NaOH) (2).}$$

$$[\alpha]_{5780}^{18} + 155 \text{ (N/2 NaOH) (2)}$$

$$[\alpha]_{5780}^{18} + 161 \text{ (2N NaOH) (2)}$$

2.4 Dissociation Constant

Ascorbic acid is a moderately strong organic acid, two ionization constants:

pK_1 4.17 and pK_2 11.57. pH = 3 (5mg/ml), pH = 2 (50 mg/ml) (4).²

2.5 Identification

- i) Solution of ascorbic acid decolorises, 2,6-dichlorophenol-indophenol solution (5).
- ii) Solution and ascorbic acid reduces silver nitrate solution immediately in the cold producing a black precipitate (5).
- iii) Dissolve 0.1 g of ascorbic acid in sufficient water to produce 100 ml and dilute 1 ml to 100 ml with 0.01M *hydrochloric acid*. The light absorption of the resulting solution exhibits a maximum only at 244 nm; A (1 per cent, 1 cm) at 244 nm, about 560 (6).
- iv) To 2 ml of a 5 per cent w/v solution add 0.5 g of *sodium hydrogen carbonate*; carbon dioxide is evolved (6).
- v) To 1 ml of a 5 per cent w/v solution add about 0.2 ml of 2M *nitric acid* and 0.2 ml of 0.1M *silver nitrate*; a grey precipitate is produced (6).
- vi) To 5 ml of a 1 per cent w/v solution add 0.05 ml of a freshly-prepared 5 per cent w/v solution of *sodium nitroprusside* and 2 ml of 2M *sodium hydroxide* followed by 0.6 to 0.7 ml of *hydrochloric acid*, added dropwise with stirring; the yellow color turns blue (6).
- vii) Specific optical rotation, in a 10 per cent w/v solution, +20.5° to +21.5° (6).
- viii) A solution (1 in 50) reduces alkaline cupric tartrate TS slowly at room temperature but more readily upon heating (7).

- ix) To 2 ml of a solution (1 in 50) add 4 drops of methylene blue TS, and warm to 40°: the deep blue color becomes appreciably lighter or is completely discharged within 3 minutes (7).
- x) Dissolve 15 mg in 15 ml of a solution of trichloroacetic acid (1 in 20), add about 200 mg of activated charcoal, shake the mixture vigorously for 1 minute, and filter through a small fluted filter, returning the filtrate, if necessary, until clear. To 5 ml of the filtrate add 1 drop of pyrrole, and agitate gently until dissolved, then heat in a bath at 50°: a blue color develops (7).
- xi) For the examination of vegetable infusions for the presence of vitamin C, the ascending - descending paper-chromatographic method of Block was used on the dinitrosazones. Many combinations of solvent were used, but a mixture of xylene and nitrobenzene (95:5) were the most satisfactory. Of these, the latter solvent furnished compact spots of such definition that treatment with alcoholic potash to reveal them was unnecessary (8).
- xii) Amounts of vitamin C down to 3 µg can be detected as dark areas on the layer exposed to short-wave UV light; brief heating to 120°C renders it fluorescent in radiation of 365 nm (9).
- xiii) The customary identification of free ascorbic acid depends on its strong reducing properties and any of the reactions known from paper chromatography may be utilized. The limit of detection with indophenol reagent (blue) is around 0.1 µg dipyridyl-iron (red) and molybdophosphoric acid (blue) are almost as sensitive; after brief heating, derivatives and decomposition products yield the colors also. Amounts of 3-5 µg can be visualized with iodoplatinate reagent (yellow) and with alkaline silver nitrate reagent (9).

2.6 Spectral Properties

2.6.1 Ultraviolet Spectrum

The UV spectrum of ascorbic acid (0.002%) in aqueous, acidic methanolic, ethanolic and alkaline solution was scanned from 200 to 400 nm using Varian Carry 119 Spectrophotometer (Fig. 1). The UV maxima are as follows:

	λ_{max} (nm)
Aqueous solution	263
Acidic solution	243
Methanol	244
Ethanol	245

Other reported data (2) are as follows:

	λ_{max} (nm)
Aqueous solution	260-265
Acidic solution (pH ₃)	245
Ethanol	245
Methanol	263
Sodium salt in aqueous solution	265

2.6.2 Infrared Spectrum

The IR spectrum of ascorbic acid as KBr-disc was recorded on a Perkin-Elmer 580B FT-spectrometer (Fig. 2). The structural assignments have been correlated with the following band frequencies (Table 1).

Table - 1: IR Characteristics of Ascorbic Acid.

Frequency Cm^{-1}	Assignment
3510	oH
3405	
3306	
1755	C = O
1670	
1110	C-O-C
1025	

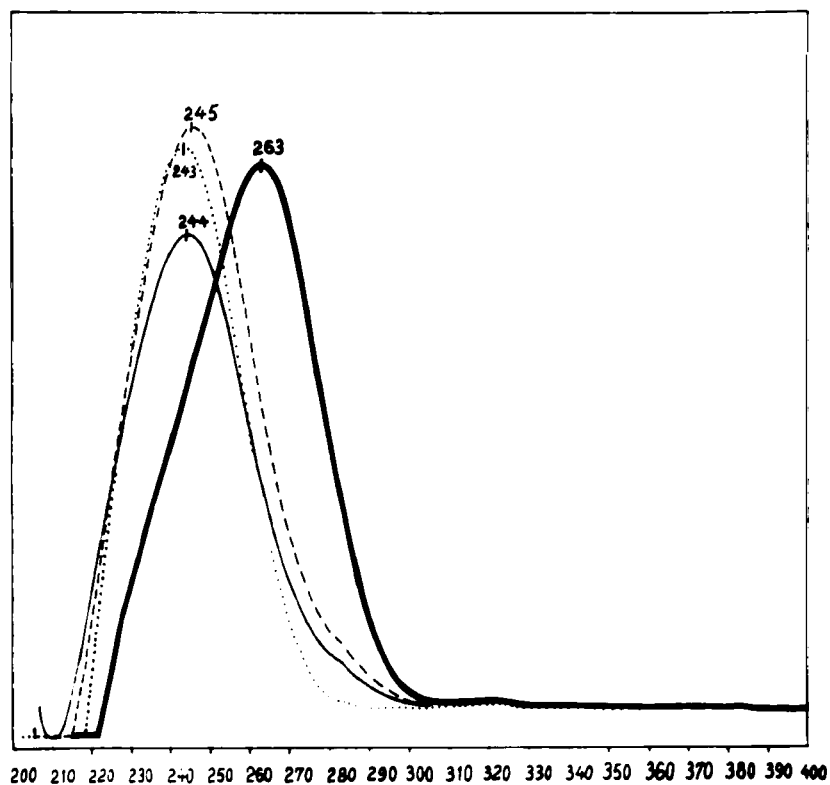


Fig. 1. UV Spectrum of Ascorbic Acid. — Ascorbic Acid in Water; --- Ascorbic Acid in Ethanol; — Ascorbic Acid in Methanol; Ascorbic Acid in Acid Solution.

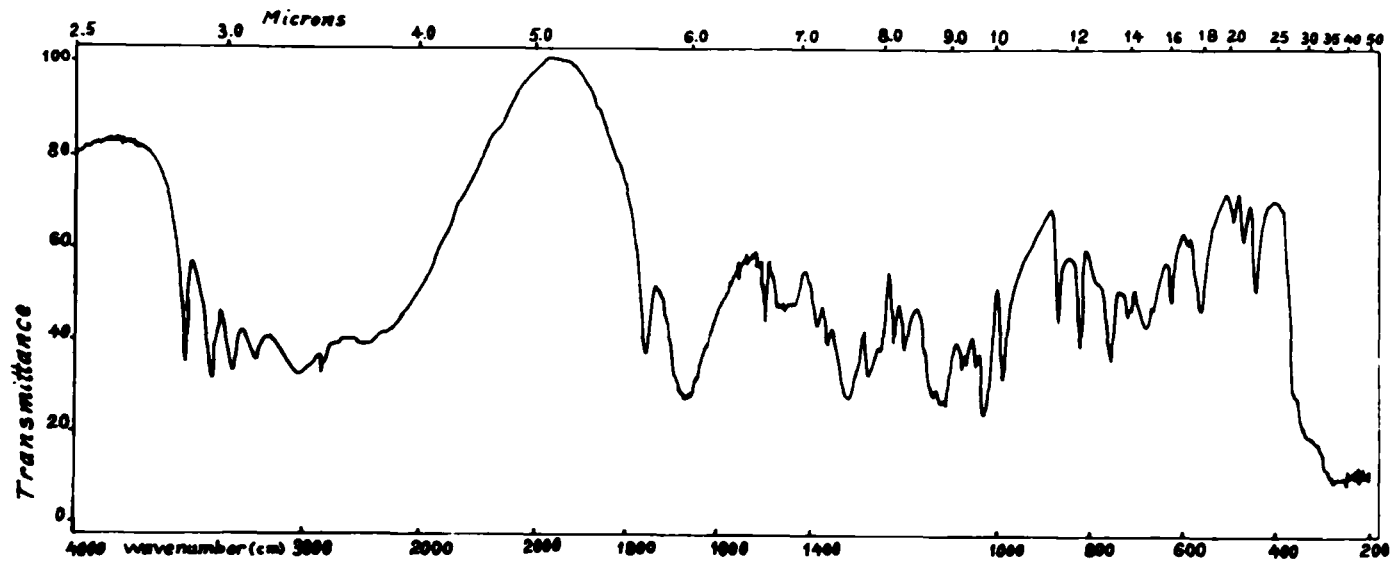


Fig. 2. IR Spectrum of Ascorbic Acid as KBr disc.

Other characteristic absorption bands are:

3208, 1500, 1390, 1372, 1320, 1275, 1250,
1222, 1200, 1140, 1075, 1068, 1045, 1025,
990, 870, 820, 755, 720, 680.

2.6.3 Nuclear Magnetic Resonance Spectra

2.6.3.1 Proton Spectra

The PMR spectra of ascorbic acid in deuterium oxide, in pyridine and in pyridine D₅ were recorded on a Varian-T-60-A, 60 MHz spectrometer using sodium-2,2-dimethyl-2-silapentane-5-sulphonate and tetramethylsilane as reference standard respectively (Fig.3, Fig. 4 and Fig. 5). The following structural assignments have been made (Table 2):

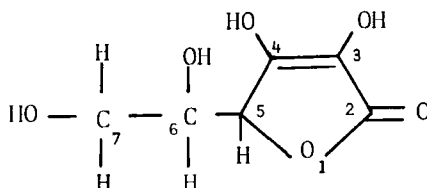


Table - 2: PMR Characteristics of Ascorbic Acid.

Chemical Shift (ppm)

D ₂ O	Pyridine	Pyridine D ₅	Assignment
4.87(d)	5.43(d)	5.36(d)	5-H
4.10(m)	4.68(m)	4.33(m)	6-H
3.77(s)	4.40(s)	4.36(s)] 7-CH ₂
3.68(d)	4.30(s)	4.23(d)	

s=singlet, d=doublet, m=multiplet.

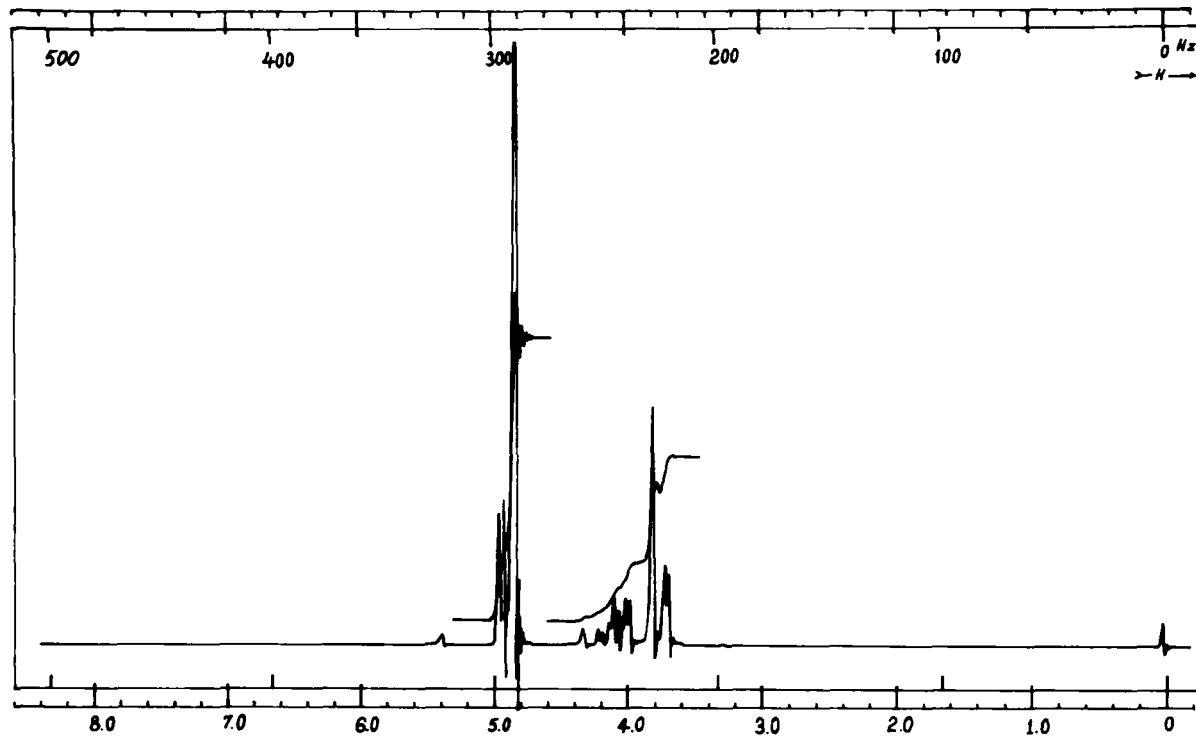


Fig. 3. PMR Spectrum of Ascorbic Acid in D₂O.

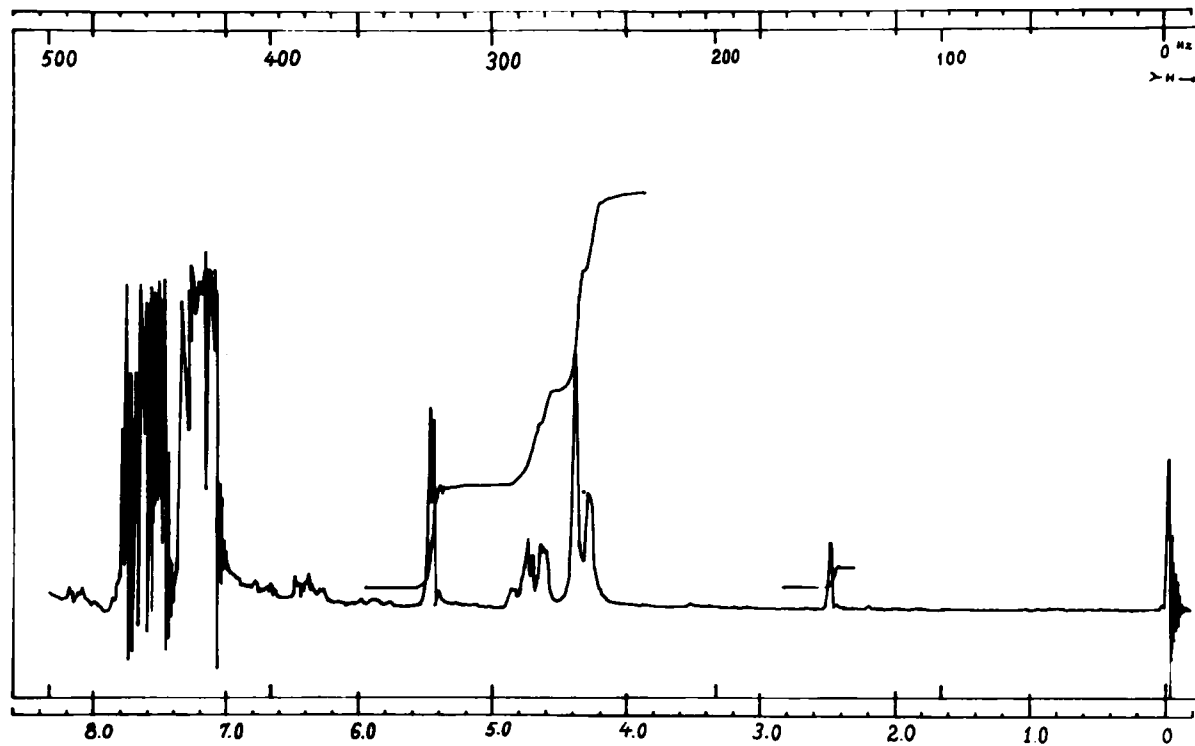


Fig. 4. PMR Spectrum of Ascorbic Acid in Pyridine.

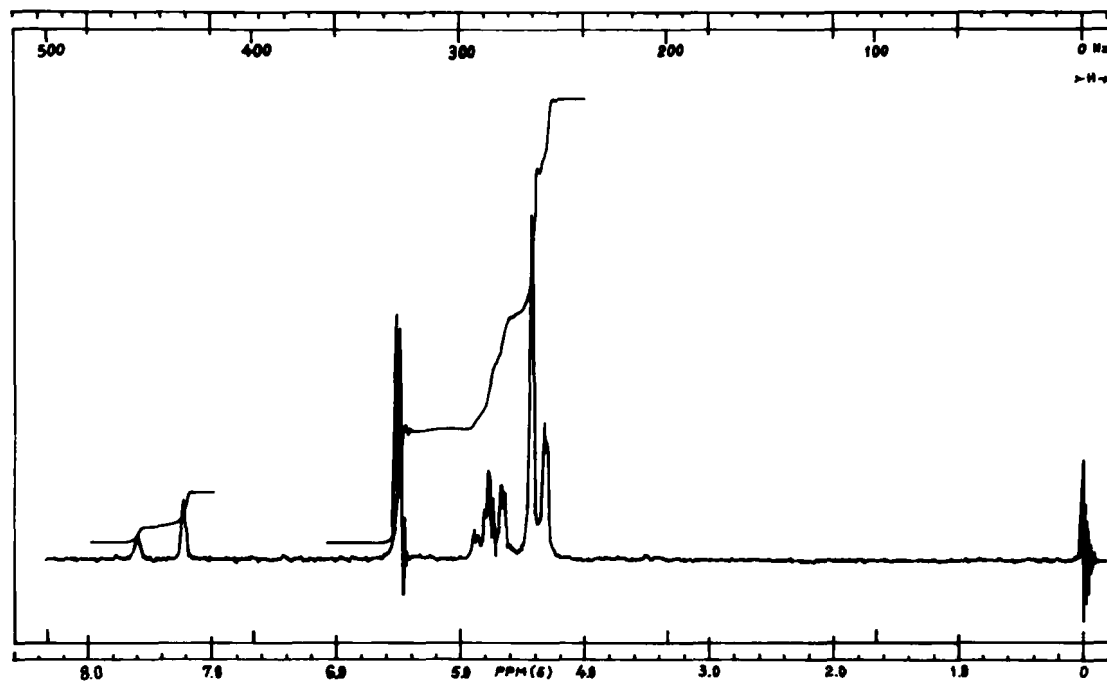


Fig. 5. PMR Spectrum of Ascorbic Acid in Pyridine D₅.

PMR data in D₂O and in a mixture of DMSO D₆ and CDCl₃ were also reported (10,11,12).

2.6.3.2 ¹³C-NMR

The ¹³C-NMR completely decoupled and off-resonance spectra are shown in Fig. 6 and Fig. 7 respectively. Both were recorded over 5000 Hz range in dimethylsulfoxide on Jeol FX-100, 100 MHz spectrometer. Using 10 mm sample tube and tetramethylsilane as reference standard, at ambient temperature. The carbon chemical shifts are assigned on the basis of the additivity principles and the proton-coupled spectrum (Table 3).

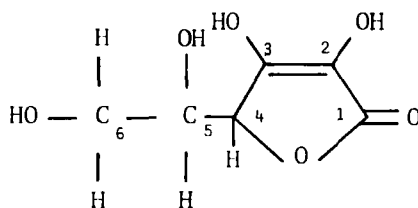


Table - 3: Carbon Chemical Shifts of Ascorbic Acid.

Carbon No.	Chemical Shift ppm
C-1	170.31(s)
C-2	117.93(s)
C-3	152.62(s)
C-4	74.56(d)
C-5	68.42(d)
C-6	61.93(t)

s=singlet; d=doublet; t=triplet

¹³C-NMR data in Water have been also reported (13).

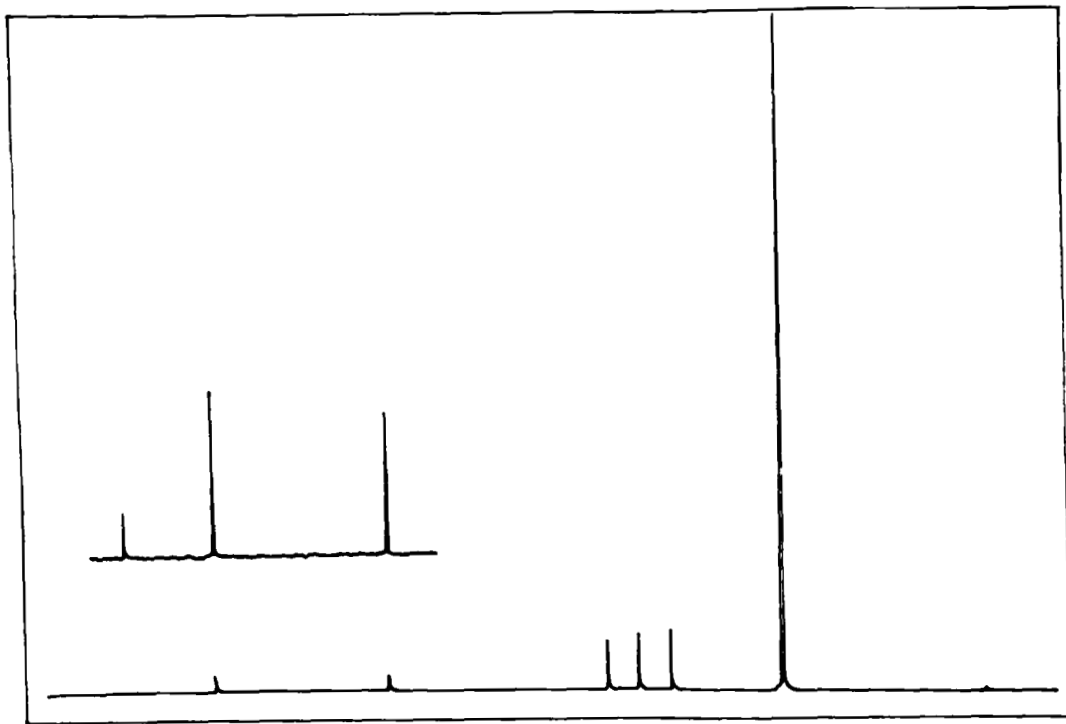


Fig. 6. ^{13}C -NMR Spectrum of Ascorbic Acid (Completely decoupled).

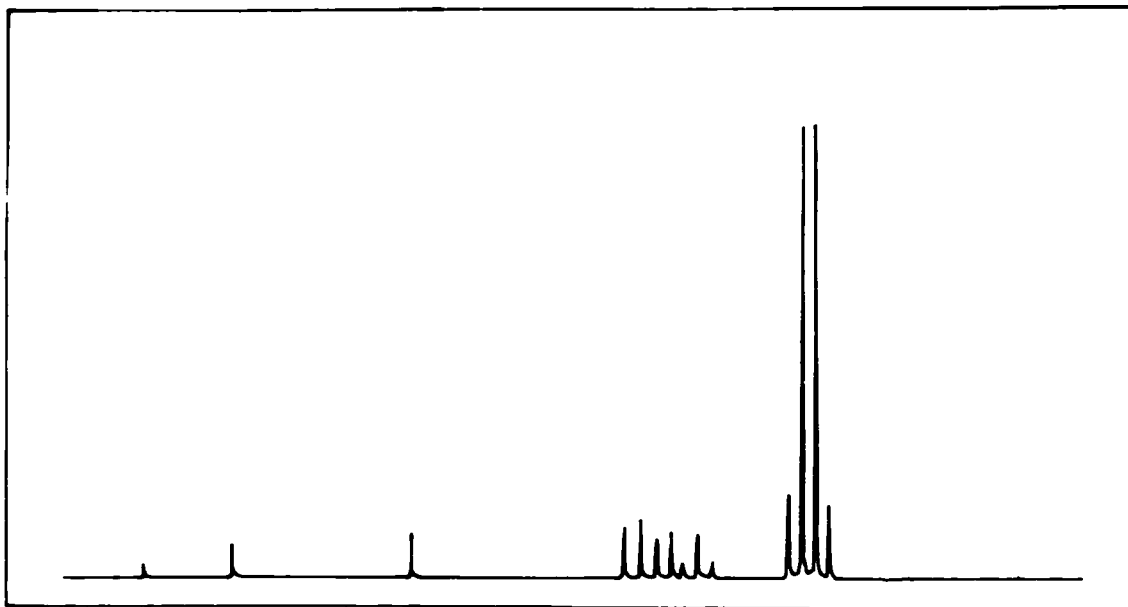
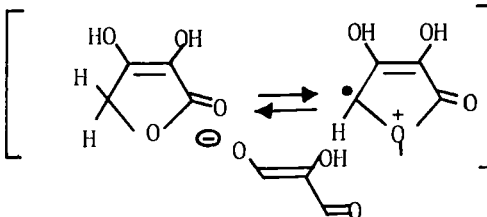
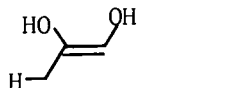
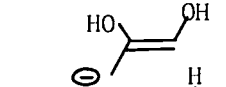
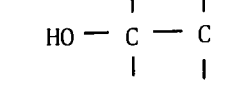
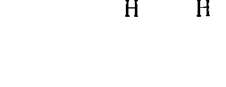


Fig. 7. ^{13}C -NMR Spectrum of Ascorbic Acid (off-Resonance).

2.6.4 Mass Spectrum

The mass spectrum of ascorbic acid obtained by electron impact ionization, was recorded on a Ribermag R-10-10 mas spectrometer equipped with direct inlet probe. The spectrum (Fig. 8) shows a molecular ion peak M^+ at m/e 176 with a relative intensity of 5.9%. The most prominent fragments and their relative intensities are shown in Table 4:

Table - 4: Prominent Mass Fragments of Ascorbic Acid.

M/e	Relative Intensity %	Fragment
177	1.0	$M + 1$
176	5.9	$M +$
116	100.0	
85	36.0	
71	24.7	
70	23.1	
61	29.7	

3. Preparation

3.1 Isolation

Many methods were reported for the isolation of ascorbic acid from plants. However, the most popular is by using freshly prepared solution of 5-6% metaphosphoric acid (14). This solution is a good extractant as well as stabilizing agent for a limited period by complexing metal ions and minimizing the rate of oxidation. It has also been claimed that ascorbic acid can be stabilized by diluted perchloric acid solution

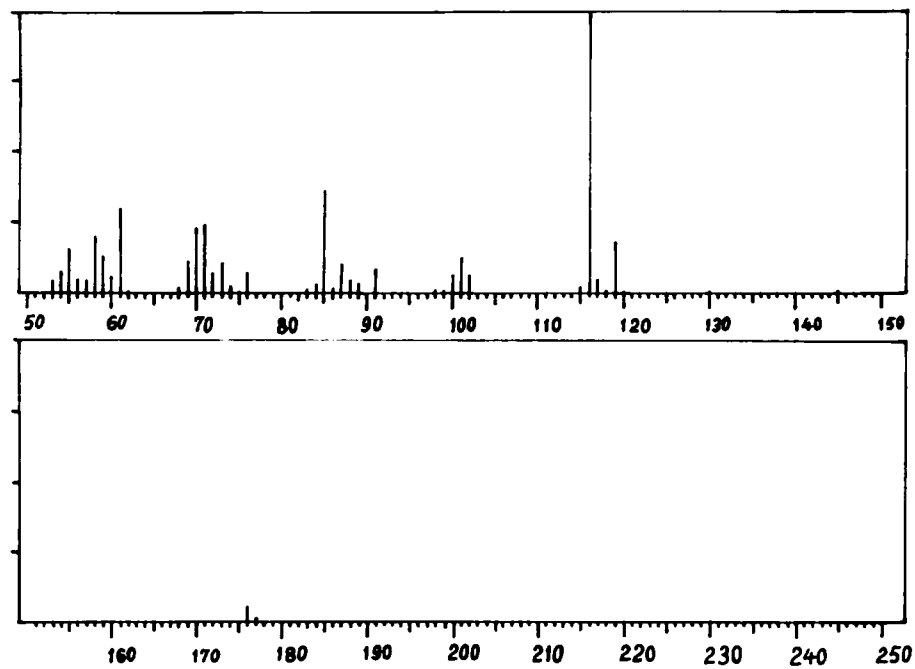


Fig. 8. Mass Spectrum of Ascorbic Acid.

or 2,3-dimercapto-1-propanol (15). An alternative method of extracting ascorbic acid from foods is by forming a slurry of the frozen material with absolute ethanol has been found to be as effective as extraction with metaphosphoric acid (16). Also a mixture of 8% acetic acid and 0.5% oxalic acid was used (17)

3.2 Synthesis

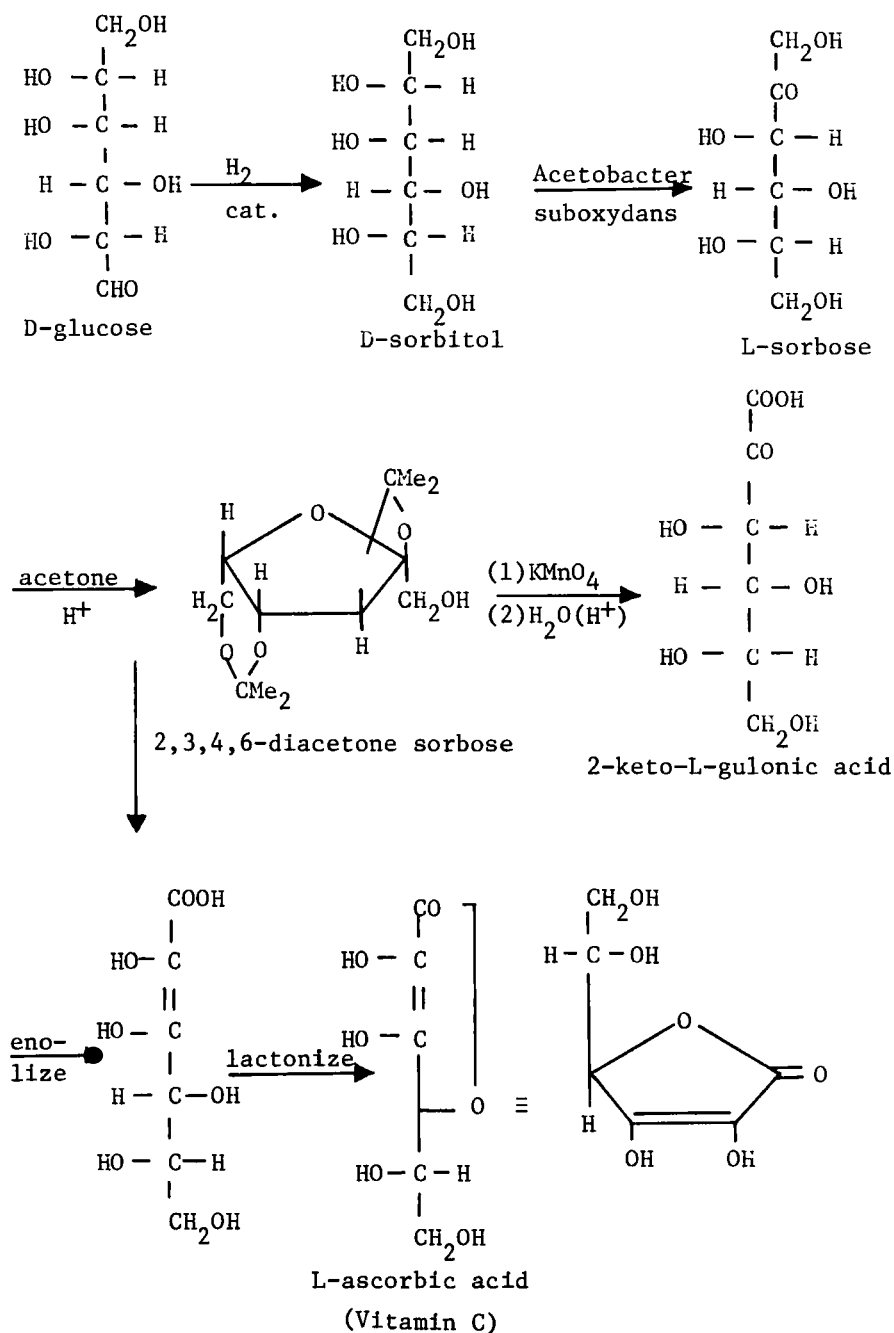
L-ascorbic acid is conventionally synthesized (18,19) by hydrogenating D-glucose to D-sorbitol. The latter is made to yields L-sorbitol by oxidation with *Acetobacter suboxydan*, this followed by introducing carbonyl group at C₁ while the L-sorbose is in the form of its diacetone derivative. The resulting diacetone-2-keto-L-gluconic acid is then heated with hydrochloric acid to give ascorbic acid (Scheme 1, p.11).

Alternative route from sorbose by oxidation with nitrogen peroxide.

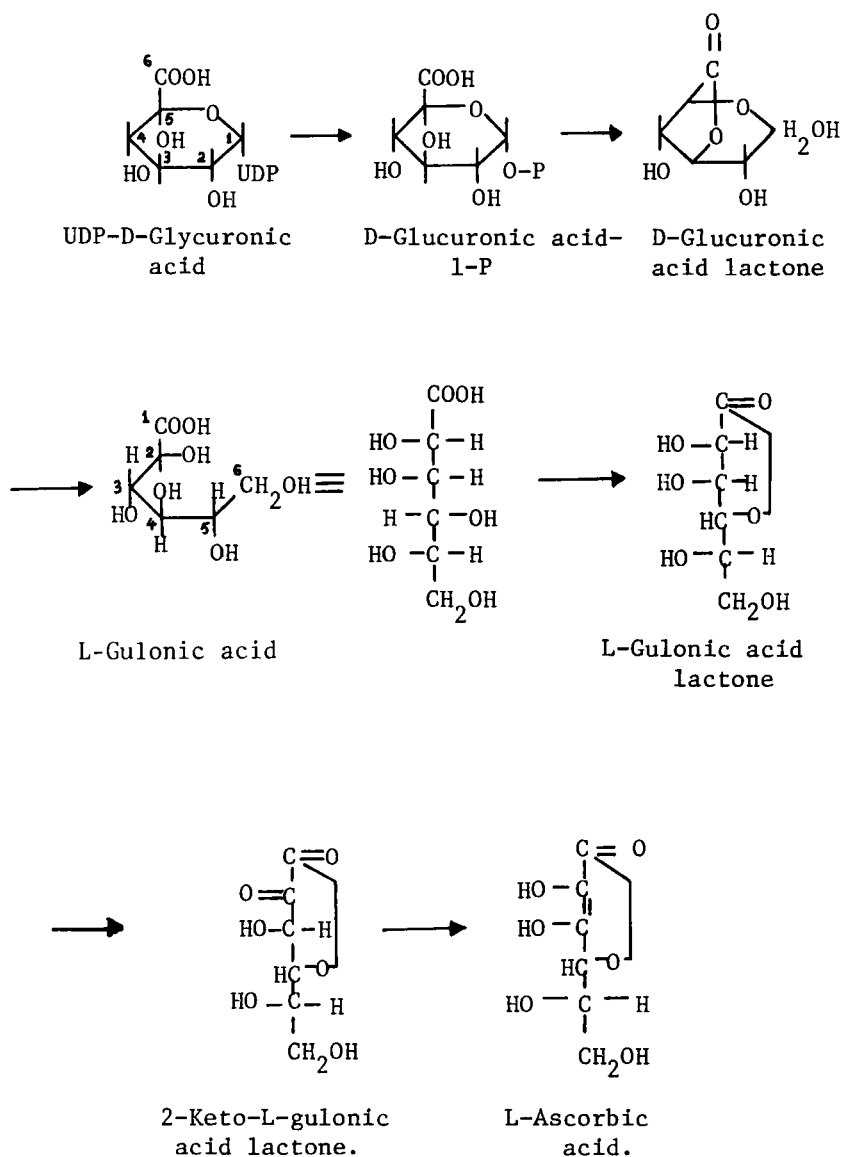
Another method for synthesizing L-ascorbic acid was reported (20), involving a one-step oxidation of 1,2-0-isopropylidene- α -D-glucofuranose to 1,2-0-isopropylidene- α -D-xylo-hexofuranurono-6,3-Lactone-5-ulose and acid treatment of the later followed by reduction.

4. Biosynthesis of Ascorbic Acid

In both plants and animals ascorbic acid is formed from D-glucuronic acid. UDP-glucuronic acid is first converted to D-glucuronic acid lactone via D-glucuronic acid-1-phosphate. This compound is then reduced at carbon atom 1 to form L-gulonic acid. (Since in the numbering of the carbon atoms of carbohydrates the most highly oxidized carbon atom is given the lowest possible number, the original carbon atom 6 of glucuronic acid becomes carbon atom 1 of gulonic acid). After the conversion of the gulonic acid to the corresponding γ -lactone, the hydroxyl group at carbon atom 2 is oxidized to a keto group. The 2-keto-L-gulonic acid lactone formed is subsequently converted to L-ascorbic acid by enolization (21). Direct conversion of D-glucuronic acid to L-gulonic acid by isomerization at carbon atom 5 has not yet been conclusively established (Scheme 2, p.12).



Scheme 1: Synthesis of ascorbic acid.



Scheme 2: The biosynthesis of L-ascorbic acid.

5. Metabolism

Ascorbic acid is readily absorbed and metabolised. However, after oral administration of large quantities, only small amounts are excreted in the urine while there is a steady rise in the level of ascorbic acid in the plasma. If the oral ingestion is continued for a sufficient period, the plasma concentration rises to a maximum, after which a rapid urinary excretion of a large part of the ingested ascorbic acid occurs (22). Ascorbic acid (AsA) and dehydroascorbic acid (DAsA) are metabolized by humans, and the levels of AsA in blood were maximum at the 4th hr after AsA administration but at 2nd hr after DAsA administration. The amount of AsA and DAsA excreted in urine in 6 hr was respectively 60 and 70% of the amount excreted in 24 hr after administration. About 30 and 40% of administered dose were excreted by men and women respectively in 24 hr. Of the vitamin excreted after administration of either AsA or DAsA, about 90 and 10% were in the form of AsA and DAsA, respectively (23).

The amino acids phenylalanine and tyrosine are not metabolized completely in vitamin C-deficient individuals. Under these conditions they are metabolized only partly and are excreted in the urine as homogentisic, *p*-hydroxyphenylpyruvic and *p*-hydroxyphenyllactic acids. It appears that vitamin C plays the role of a coenzyme in the metabolism of tyrosine through its deaminated product, because scorbutic liver slices cannot metabolize this amino acid in the absence of this vitamin. Vitamin C in adequate amounts delays the oxidation of epinephrine by the body (24).

6. Daily Requirement!

Ascorbic acid is needed in daily quantities of about 70 mg to sustain full stamina, and is an essential nutrient for human beings. In the case of insufficiency the symptoms of scurvy appears (21).

7. Mode of Action

We have as yet no complete understanding of the mode of action of ascorbic acid. It is, however, known that this substance is involved in certain hydroxylation reactions which are catalysed by mixed function oxygenases in the reduction of folic acid to tetrahydrofolic acid as well as well in the regulation of the redox equilibrium between Fe^{2+} and Fe^{3+} and Cu^{+} and Cu^{2+} (21).

8. Vitamin Defficiency

Patients placed on a diet deficient in vitamin C exhibited the following: 1) 10 days, plasma fell to a low level; 2) 30 days plasma level was zero; 3) 13 weeks, first clinical evidence of scurvy; 4) 132 days, hyperkeratotic papules developed; 5) 141 days, wounds failed to heal and 6) 162 days, perifollicular hemorrhages of scurvy developed; ascorbic acid value of white cell platelets fell to zero. Loss of weight occurred, accompanied by lowered blood pressure (24).

9. Methods of Analysis

9.1 Titrimetric Methods

British Pharmacopoeia 1973

The B.P. 1975 has described the assay as follows:

Weigh and powder 20 tablets. Dissolve a quantity of the powder equivalent to 0.15 g of ascorbic acid as completely as possible in a mixture of 30 ml of water and 20 ml of *dilute sulphuric acid* and titrate with 0.1N *ammonium ceric sulphate*, using *ferroin sulphate solution* as indicator. Each ml of 0.1N *ammonium ceric sulphate* is equivalent to 0.008806 g of $C_6H_8O_6$.

British Pharmacopoeia 1980

The B.P. 1980 has described the assay as follows:

Dissolve 0.2 g in a mixture of 80 ml of freshly boiled and cooled water and 10 ml of M *sulfuric acid*. Titrate with 0.05 M *iodine VS* using 1 ml of *starch solution* as indicator until a persistent blue color is obtained. Each ml of 0.05M *iodine VS* is equivalent to 0.00881 g of $C_6H_8O_6$.

United States Pharmacopoeia 1980

a) Ascorbic Acid: Dissolve about 400 mg of ascorbic acid, accurately weighed, in a

mixture of 100 ml of carbon dioxide-free water and 25 ml of *2N sulfuric acid*. Titrate the solution at once with 0.1 *N iodine VS*, adding 3 ml of starch TS as the end-point is approached. Each ml of 0.1 *N iodine* is equivalent to 8.806 mg of $C_6H_8O_6$.

- b) Ascorbic acid injections: Transfer to a 100-ml volumetric flask an accurately measured volume of ascorbic acid injection, equivalent to about 50 mg of ascorbic acid and previously diluted with water if necessary. Add 20 ml of metaphosphoric-acetic acids TS, dilute with water to volume, and mix. Accurately measure a volume of the dilution, equivalent to about 2 mg of ascorbic acid, into a 50-ml conical flask, add 5 ml of metaphosphoric-acetic acids TS, and titrate with standard dichlorophenol-indophenol solution until a rose-pink color persists for at least 5 seconds. Correct for the volume of the dichlorophenol-indophenol solution consumed by a mixture of 5.5 ml of metaphosphoric-acetic acids TS and 15 ml of water. From the ascorbic acid equivalent of the standard dichlorophenol-indophenol solution calculate the ascorbic acid content in each ml of the injection.

Various other titrimetric methods of assay of vitamin C in vegetable tissues, whether as ascorbic acid or as total vitamin C (ascorbic plus dehydroascorbic acids), were studied and compared. For the extraction of the vitamin, an aqueous mixture of 8 per cent, acetic and 0.5 per cent oxalic acids was used instead of metaphosphoric acid. Hot and cold extractions gave practically the same results, except with hard, dry tissues, which required hot extraction. To effect removal of colloidal matter, which interferes with filtration and clarification of the extract, it is considered advisable to add 25 to 50 per cent of ethanol. In the presence of ethanol causes an error of at least 2 to 12 per cent. To establish (visually) the titration end-point in the indophenol method, it is recommended that a standard having the same composition as the sample under titration

but previously oxidised with iodine and mercuric acetate be employed. This generally enables 93 to 100 per cent of vitamin C experimentally added to be determined. In the method of determination with methylene blue, the ratio of methylene blue used to vitamin C content decreases as the latter value increases. This decrease of the ratio is small; to obtain satisfactory results, the aliquot titrated should not contain >0.04 mg of vitamin C. Visual determination of the end-point with the aid of a standard is again recommended. The iodimetric method, with potassium iodate was also studied. The conclusions drawn are: 1) that the indophenol method is sufficiently accurate, is the most simple method and has the widest sphere of application; 2) that the methylene blue method gives results 3 to 10 per cent lower than the indophenol method and 3) that the iodimetric method gives results 20 to 40 per cent higher (17).

Reduction is effected by adding M sodium sulphide solution acidified with HCl, and removing the excess of sulphide with M ethanolic mercuric chloride solution. Reduction is complete in 10 to 15 min. Clear filtrates are readily obtainable for titration with dichlorophenol-indophenol (25).

Simple methods of determining ascorbic acid, thiamine and nicotinic acid are applied to mixtures containing these vitamins with various pharmaceutical preparations. Ascorbic acid can be determined iodimetrically in the presence of calcium lactate, phytin glucose calcium glycerophosphate, caffeine sodium benzoate, nicotinic acid, amidopyrine or thiamine. For mixture containing ascorbic acid and nicotinic acid, the ascorbic acid is determined iodimetrically and the total acid is

then titrated against 0.1 *N* solution of hydrochloric acid with phenol red as indicator. Thiamine is determined in the presence of ascorbic acid by a modification of the U.S.S.R. Pharmacopoeia VIII argentimetric method (26).

The platinum - tungsten bimetallic electrode system is applicable to the quantitative iodimetric determination of ascorbic acid according to the B.P. 1953. The equivalence point is given by the very sharp break in the titration curve. The error, ± 0.0002 on samples of 0.05 to 0.15 g is much less than for the usual volumetric procedure (27).

Other titrimetric method for the determination of ascorbic acid is by involving two stage oxidation by potassium iodate (28).

9.2 Spectrophotometric Method

9.2.1 Colorimetric

An assay method described for ascorbic acid involves the reaction with diazotised 4-methoxy-2-nitroaniline in acid medium, and subsequent development of a blue color in alkaline solution. This color, with a maximum absorbancy at 570 nm, is compared with standards in suitable photo-electric colorimeter. It can be carried out directly, *e.g.*, in the presence of dehydro-ascorbic acid and all other vitamins. Its sensitivity permits the determination of quantities down to 0.5 mg with a low limit of 10 μ g per ml, when a 50-ml sample aliquot is used (29a).

9.2.2 Ultraviolet

In aqueous solution ascorbic acid is characterised by a single very intense band with its head at 260-265 nm. The molecular extinction coefficient is approximately 7000 for solutions containing about 2 mg/100 cc. In stronger solutions (ca. 50 mg per 100 c.c.) wide deviations from Beer's law are encoun-

tered. But for concentrations ranging between 0.5 and 2.5 mg per 100 c.c. Beer's law holds with sufficient exactitude to permit of the use of spectrophotometric measurements for quantitative estimations of concentrations. The intensity of the band diminishes rapidly, falling to half value in a few hours (decomposition of ascorbic acid by oxidation (2)).

Other color reactions have been proposed as a basis for measuring ascorbic acid: reaction with diazotised *p*-aminobenzoic acid to produce a pink color and the interaction of ascorbic acid with dimethoxydiquinone to form a reddish-violet color which is measured spectrophotometrically at 510 nm (29b).

Ascorbic acid, cystine, thioglycollic acid, α -mercaptopropionic acid and sodium mercaptobutane sulphonate are determined by the fluorescence produce by the reduction of sodium 1:2-naphthaquinone-4-sulphonate (Folin's reagent) in u.v. The use of *p*-chloromercuribenzoic acid in the determination of ascorbic acid with 2,6-dichlorophenol-indophenol below pH3-5 is recommended because of the extent of spontaneous fading of indophenol that occurs. *p*-Chloromercuribenzoic acid does not affect the reaction between indophenol and ascorbic acid, but it inhibits almost entirely the decolorisation of indophenol by various interfering substances (31).

9.2.3 Spectrofluorimetric

The reaction of dehydroascorbic acid with *o*-phenylenediamine to give a fluorescent quinoxaline was used as the basis of an assay to determine γ quantities of ascorbic and dehydroascorbic acids. The development of the fluorescent derivative of the vitamin is prevented by forming a boric acid-dehydroascorbic acid complex prior to addition of the diamine solution. This provides a means of differentiating between the fluorescence from the vitamin and that from possible interfering substances. When applied to pharmaceutical preparations, beverages and special dietary foods,

the method shows a high degree of specificity. No interfering substances were found (32).

A rapid colorimetric determination of ascorbic acid (10 to 200 mg per 100 g) with the sodium salt of 2,6-dichlorophenol-indophenol was studied. An amyl acetate extract of 2,6-dichlorophenol-indophenol has max. absorption at 525 nm the extinction of which remains unchanged for 4 hrs. The sample is extracted with 2 per cent metaphosphoric acid and diluted to produce a 0.4 to 1.0 mg per cent solution of ascorbic acid. 2,6-dichlorophenol-indophenol solution (5 ml in water) is added to the sample solution which is rapidly extracted with amyl acetate for the extinction to be measured. Another 5-ml portion of the 2,6-dichlorophenol-indophenol solution is added to metaphosphoric acid and similarly treated. The amount of ascorbic acid is determined from the difference in the extinction of the two extracts, which is proportional to the concentration of ascorbic acid up to 4 mg per 100 ml (33).

Other method depends on the reduction of ferric chloride by ascorbic acid and the colorimetric determination of the ferric chloride by means of reaction with α -dipyridyl; the reaction is carried out in the presence of phosphoric acid to eliminate interference by reduction. A solution (1 ml) containing \approx 0.02 mg of ascorbic acid is treated with 0.3 ml of 85 per cent. Phosphoric acid to give a pH of 1 to 2, 5 ml of 0.5 per cent aqueous α -dipyridyl solution and 1 ml of 1 per cent ferric chloride. The method has been applied to orange juice, honey and urine; it is sensitive to 5 μ g of ascorbic acid per ml (34).

A direct method for the determination of vitamin C without removal of the reagent, 2,4-dinitrophenylhydrazine, is applied to blood and urine. The ascorbic acid content is calculated from a chart (35).

Ascorbic acid can be semi-quantitatively estimated with the comparison method after color reaction with molybdophosphoric acid.

The red zone of dehydroascorbic acid-DNP is scraped off, eluted with 85% sulphuric acid, filtered or, better, centrifuged, and the light absorption of the solution measured at 520 to 525 nm against water as blank. The analysis result is worked out with the help of a standard solution which is treated identically (9).

9.3 Turbidimetric Method

This method (36) is used for the determination of ascorbic acid in foods, by the reaction of selenious acid with ascorbic acid and stannous ions at low pH and room temperature.

9.4 Chromatographic Methods

9.4.1 Paper Chromatography

Methods used for the separation of ascorbic acid by paper chromatography are shown in Table 5 (p. 21).

9.4.2 Gas-Liquid Chromatography

Gerstl and Ranfft (42) extracted food with metaphosphoric acid, separated the ascorbic acid on a column of cellulose and formed the trimethylsilyl ether derivative by reaction with N.O-bis-(trimethylsilyl) acetamide before chromatographing on a column of Gas Q containing 3% SE-30.

9.4.3 High Pressure Liquid Chromatography

Packla and Kissinger (43), used a strong anion resin and elution with pH 4.75 buffer solution, has been applied to the determination of ascorbic acid in milk products, baby foods, fruit juice concentrates, whole fruits and fortified cereals. The procedure was not directly suitable for measuring dehydroascorbic acid. However, Sood et al (44) used HPLC in

TABLE - 5 Methods used for separating ascorbic acid by paper chromatography

Chromatogram	Solvent System	R _F Value	Reagent	Application	Reference
Paper Chromatography.	1) n-butanol saturated with water + oxalic acid. 2) Phenol saturated with water + oxalic acid.		2,6-dichlorophenol-indophenol with subsequent colorimetry.	Plant and animal tissues.	37
Paper(Schleicher and Schull No. 602).	1) 50% Methanol. 2) Water-n-butanol-glacial acetic acid (50:40:14:5)	0.7-0.8 0.6-0.7	1) 1% silver-nitrate solution in 10% Aqueous ammonia. 2) 0.005N iodine solution in 0,04% starch solution. 3) 0.04% aqueous solution of 2,6-dichlorophenol-indophenol. 4) U.V.	Lemon juice white, red and black-currents. tomatoes and green pappers.	38
Paper Chromatography.	butanol-acetic acid-water (4:1:5)	0.36	ammonium-molybdate solution.		39
Paper Chromatography.	n-butanol-acetic acid-water (4:1:5)		Alkaline tetrazolium salts.	Method for detection of 15µg quantity.	40

the reverse phase ion-pairing mode to determine ascorbic acid in food with tridecyl ammonium formate as counter-ion.

9.5 Enzymatic Method

A new enzymatic method based on the oxidation of the ascorbic acid to dehydroascorbic acid with ascorbic oxidase permit assaying for ascorbic acid and dehydroascorbic acids in vegetable extracts (45).

9.6 Polarographic Method

Ascorbic acid can be analysed in a variety of mixtures and multivitamins preparations by cathode ray reverse sweep polarography (46).

9.7 Chronometric Method

The reduction oxidation couple between ascorbic acid oxidation and reduction of the semiquinoid form p-phenylenediaimine is the basis of a new chronometric assay of vitamin C.

Ascorbic acid interrupts formation of the colored product by coupling its oxidation to reduction of the semiquinoid proceeds to dye form (47).

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CAPTOPRIL

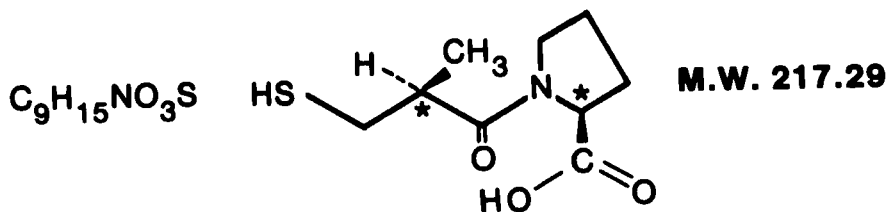
Harold Kadin

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1. Description

1.1 Name, Formula, Molecular Weight

Captopril, Capoten®, or Lopirin® is 1-(3-mercapto-2-D-methyl-1-oxopropyl)-L-proline (S,S) with Chem. Abstr. Registry Number 62571-86-2.



The asterisks indicate the two S,S optically active centers.

1.2 Appearance, Color, Odor

Captopril is a white to off-white crystalline powder with a slight mercaptan odor.

2. History

The captopril story began in 1971 with a report (1) on the isolation and synthesis of teprotide, an antihypertensive nonapeptide from the venom of a Brazilian Pit Viper. This venom peptide was hypotensive through inhibition of an exopeptidase, known as the angiotensin converting enzyme (ACE). The latter performs dipeptide scission at the carboxyl end of the decapeptide, angiotensin I to yield angiotensin II, the most powerful natural vasoconstrictor known. ACE further potentiates hypertension through scission-inactivation of the nonapeptide vasodilator, bradykinin. Clinical investigations with both teprotide (2) and captopril (3) have implicated ACE as the key enzyme in human hypertensive diseases.

The first ACE inhibitor shown to be clinically efficacious against hypertension was the synthetic venom peptide, teprotide (2).

However teprotide was expensive and only effective parenterally. A simpler, orally effective ACE inhibitor was desired.

Analogues of the snake venom hypotensive peptides were quantitatively rated for their in vitro inhibition of ACE (4) and for their effect on the contractile properties of guinea pig ileum (5). The ratings aided construction of a hypothetical active site for ACE (Figure 1). This model was based primarily on the similarity of the enzymatic properties of ACE to those of carboxypeptidase A (CASE A) even though the latter yields amino acids rather than the dipeptides of the former. Further, the active site of CASE A, like ACE contains zinc but unlike that of ACE had been structurally characterized, in the crystalline state, by x-ray diffraction (6). Thus the elaboration of the hypothetical ACE active site and the possibility of a simple ACE inhibitor were stimulated by a 1973 report (7) on, D-2-benzylsuccinic acid, a simple inhibitor, of CASE A.

Specific points of inhibitor attachment within the real CASE A site were extrapolated to that within the hypothetical ACE site (8). On the basis of these extrapolations, synthesis of simple ACE inhibitors were initiated in 1974. As each of a great number of candidates was quantitatively rated in the aforementioned in vitro screens (previously developed for the venom peptide studies), the hypothetical site was verified and refined. In the refined model schematically represented in Figure 1, three dimensional amino acid configurations within the catalytic site provided suitably spaced subsites or multiple points of attachment to substrate or inhibitor. The semi-circular clefts in the figure represent hydrophobic subsites which may interact with the lipophilic side chains of inhibitor or substrate. The latter are also putatively bound to the ACE site via the X-H group at their nonscissile terminal peptide bond. As indicated, one of the first simple ACE inhibitors was patterned after all of the venom peptide inhibitors in sharing a terminal proline (succinyl-L-proline). The choice of proline was also influenced by the superior biological stability exhibited by teprotide with

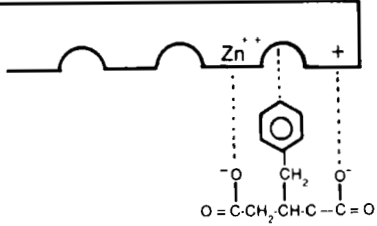
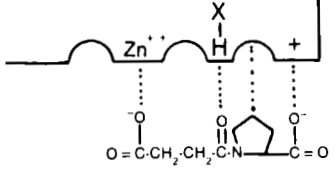
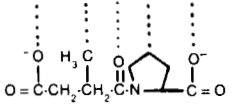
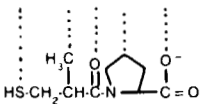
ENZYME	INHIBITOR	RELATIVE IN VITRO INHIBITION
<p>CARBOXYPEPTIDASE A</p>  <p>$\text{O}=\text{C}-\text{CH}_2-\text{CH}_2-\text{CH}(\text{C}_6\text{H}_5)-\text{C}=\text{O}$</p>	D-2 BENZYL SUCCINIC ACID	—
<p>ANGIOTENSIN-CONVERTING ENZYME</p>  <p>$\text{O}=\text{C}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{C}(=\text{O})-\text{N}-\text{PROLINE}-\text{C}(=\text{O})-\text{O}^-$</p>	SUCCINYL-L-PROLINE	1
 <p>$\text{O}=\text{C}-\text{CH}_2-\text{CH}_2-\text{CH}(\text{CH}_3)-\text{C}(=\text{O})-\text{N}-\text{PROLINE}-\text{C}(=\text{O})-\text{O}^-$</p>	D-2-METHYL SUCCINYL-L-PROLINE	15
 <p>$\text{HS}-\text{CH}_2-\text{CH}_2-\text{CH}(\text{CH}_3)-\text{C}(=\text{O})-\text{N}-\text{PROLINE}-\text{C}(=\text{O})-\text{O}^-$</p>	CAPTOPRIL	14000

Fig. 1. Key steps in the design of a specific inhibitor of the angiotensin converting enzyme.

its two terminal prolines (9). In an emulation of D-2-benzylsuccinic acid, but with appropriate lengthening of the chain, the nitrogen and carbonyl of the penultimate "venom peptide" amide linkage were substituted, respectively, with a peptidase-inhibitory methylene and a highly anionic, zinc binding carboxyl. As indicated in Figure 2 the analogy was then extended to the better snake venom inhibitors bearing a penultimate alanine in addition to the terminal proline (D-2-methylsuccinyl-L-proline). The largest increase in inhibitory activity (about 14 thousand fold over succinyl-L-proline) was obtained when the zinc binding carboxyl was replaced with a thiol which has considerably greater affinity for zinc. The snake venom peptide, teprotide, does not have comparable affinity for zinc, however its binding is enhanced by additional interactions of its amino acid side chains with "clefts" on the enzyme beyond the active peptidase site (10).

In short, the orally active antihypertensive drug, captopril, was announced to the scientific community in 1977 (11). It was made generally available for treatment of hypertensive diseases in 1981 (12,84). Clinical investigations (13) suggest it is also highly efficacious in the treatment of congestive heart failure. Its uniquely designed highly specific affinity for the active site of ACE has resulted in a high ratio of clinical success with a relatively low index of side effects. It has been effective where conventional antihypertensive therapies fail or have an untoward number of side effects.

3. Synthesis

A process (14) is presented (Figure 2) in a chemical reaction sequence which follows this brief description.

Methacrylic acid (I) is condensed with thio-lacetic acid (II) to give racemic 2-methyl-3-acetylthiopropionic acid (III). L-proline is then acylated with the acid chloride (IV) of the thio-ester (III). The resulting proline thioester (VI) is resolved from its R,S isomer by aqueous crystallization. Saponification of compound VI with sodium hydroxide affords the sodium salt of captopril which after acidification yields captopril (VII).

4. Physical Properties

4.1 Spectral Properties

4.11 Infrared Spectra

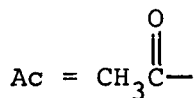
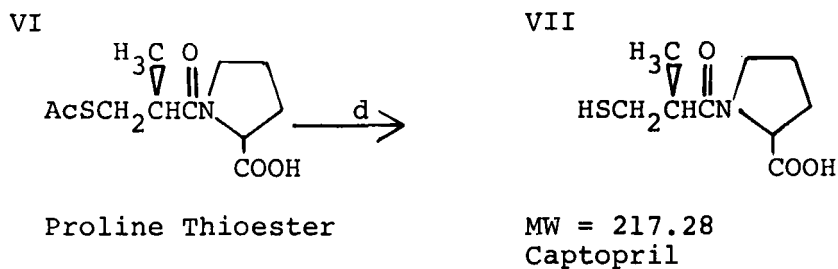
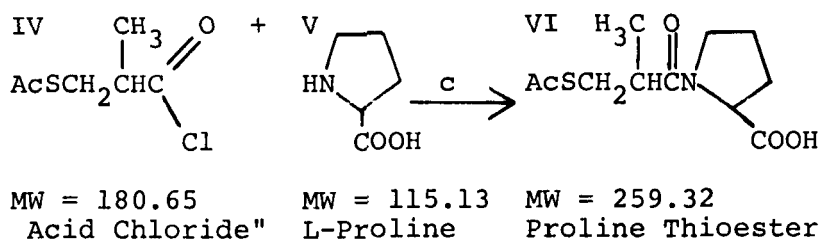
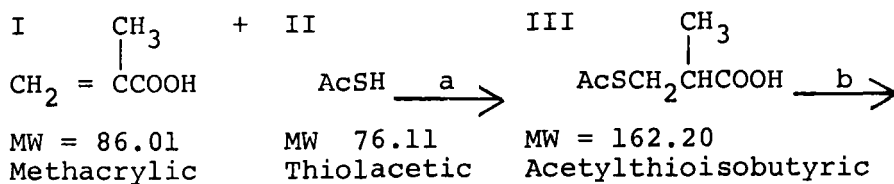
The infrared spectrum of captopril in chloroform is presented in Figure 3 and as a KBr pellet in Figure 4. The infrared spectrum in the latter indicates the presence of the following frequencies and their structural assignments (15).

<u>cm⁻¹</u>	<u>Assignment</u>
1750 } 1725 }	C = O (COOH group)
1640	C = O (amide)
2560	S - H

Differences in the fingerprint regions (1350-900 cm⁻¹) of the mineral oil mull infrared spectra of batches 3 and 4 (Figure 5 and 6, respectively) indicate that the low melting batch 3 and the high melting batch 4 are polymorphs (see Section 4.21).

Figure 2

Chemical Reaction Schematic Diagram



- (a) Reflux (b) SOCl_2 , DMF, Distillation
 (c) $\text{H}_2\text{O} + \text{NaHCO}_3$, CH_2Cl_2 Wash, HCl,
 crystallization VI.
 (d) $\text{H}_2\text{O} + \text{NaOH}$, HCl, CH_2Cl_2 extraction

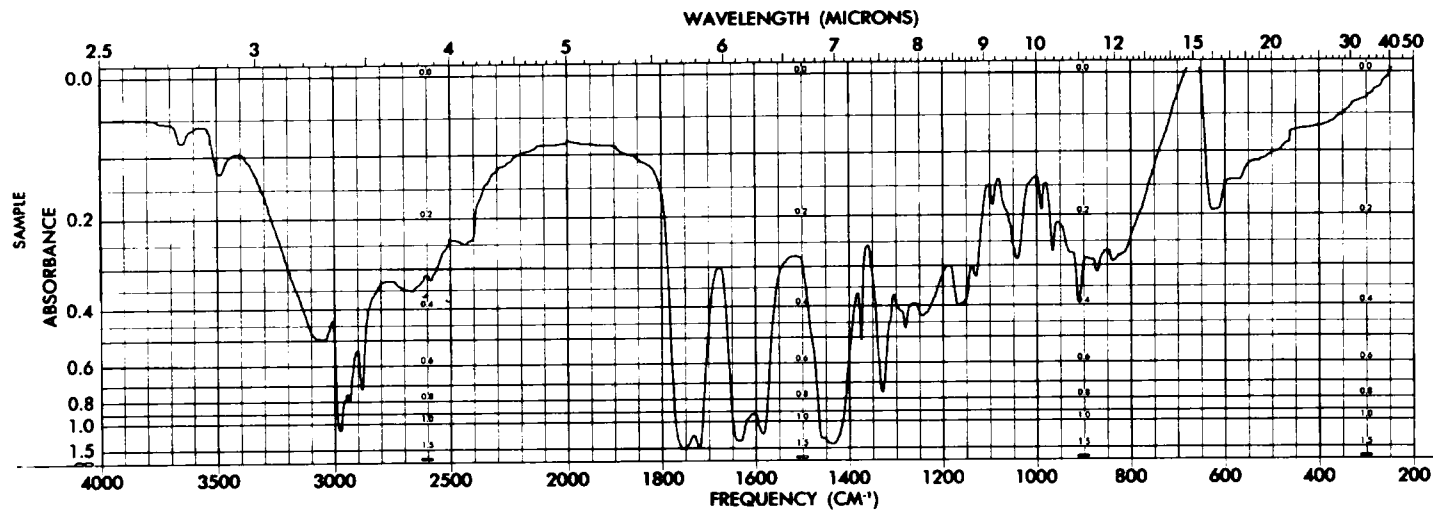


Figure 3. Infrared Spectrum of Captopril House Standard in Chloroform Solution

Instrument: Perkin-Elmer, Model 621

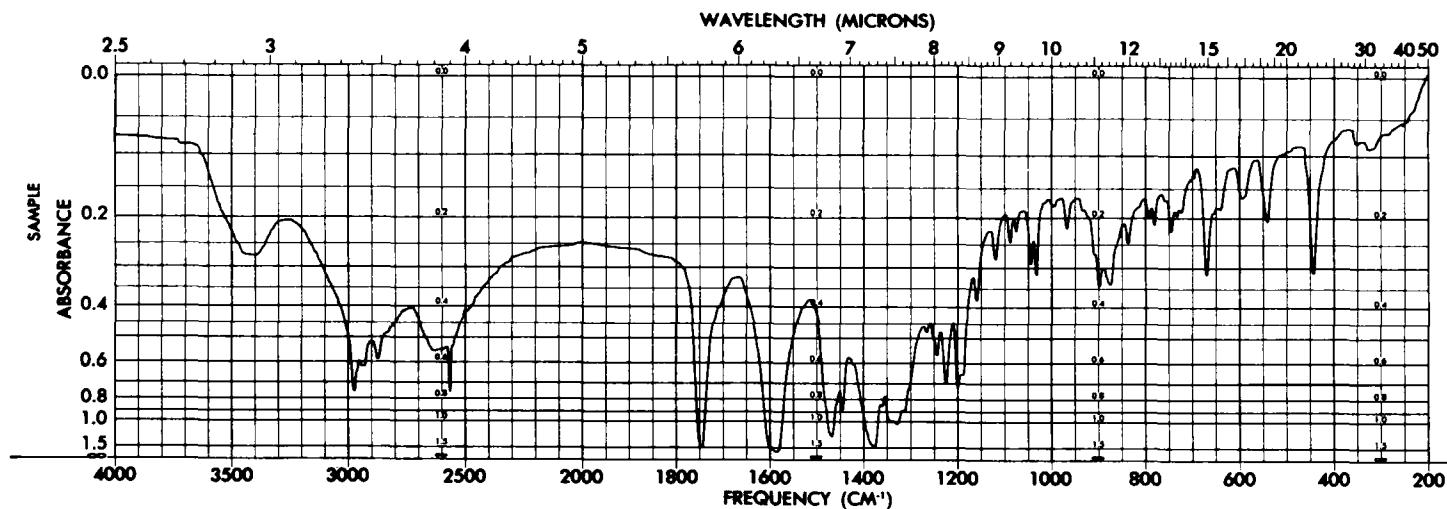


Figure 4. Infrared Spectrum of Captopril House Standard in KBr Pellet

Instrument: Perkin-Elmer, Model 621

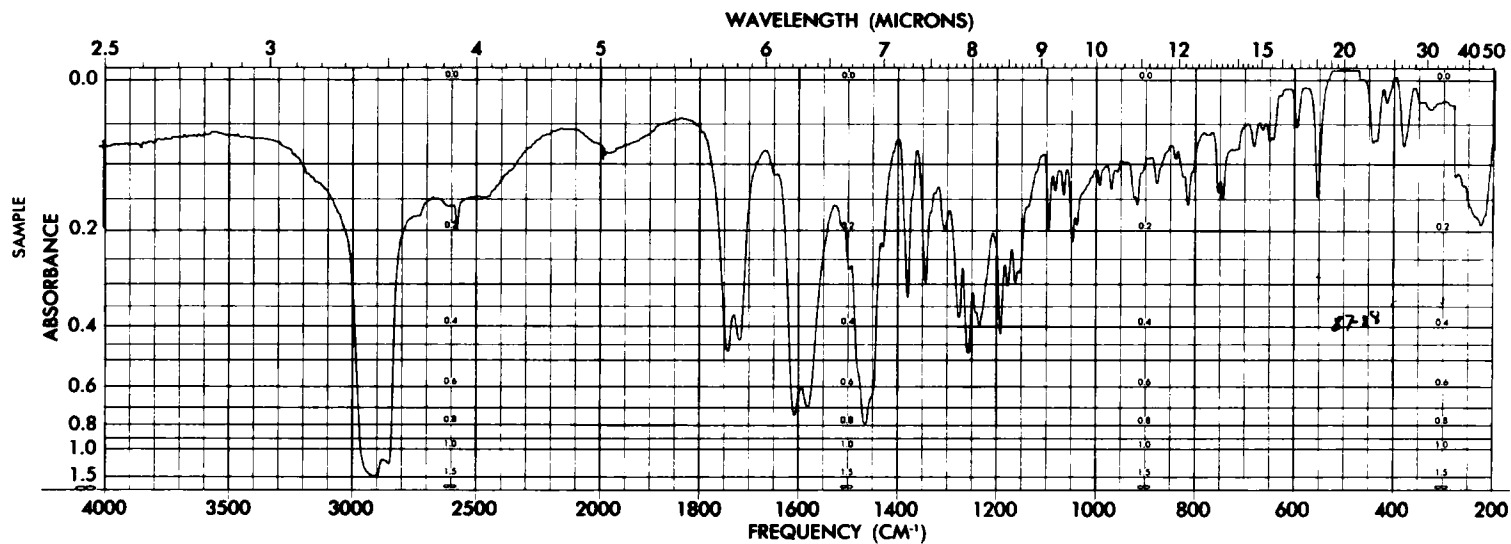


Figure 5. Infrared Spectrum of Captopril, Batch 3, Mineral Oil Mull

Instrument: Perkin-Elmer, Model 621

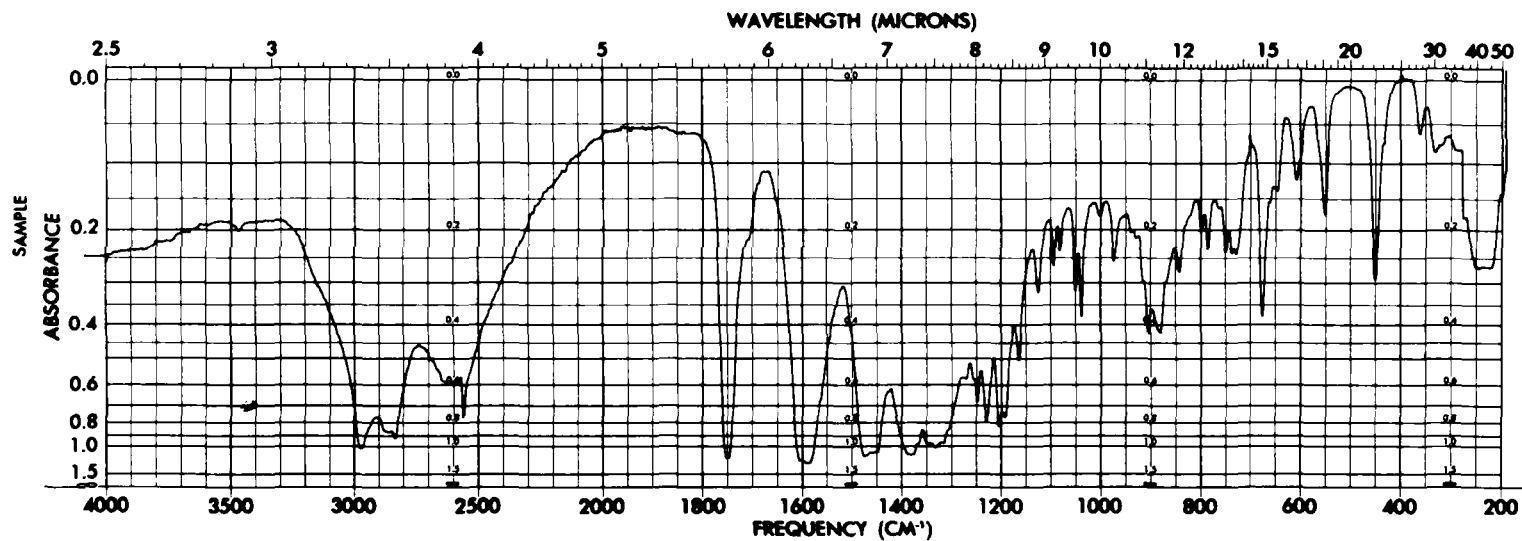


Figure 6. Infrared Spectrum of Captopril, Batch 4, Mineral Oil Mull

Instrument: Perkin-Elmer, Model 621

4.12 Nuclear Magnetic Resonance Spectra

The 270 MHz ^1H -NMR spectrum of captopril in CDCl_3 is shown in Figure 7. The spectrum was obtained from the University of Chicago courtesy of Professor Josef Fried. Spectral assignments are shown in Table 1.

The ^{13}C -NMR of captopril in CD_3OD is shown in Figure 8. The spectrum was obtained on a Varian Associates XL-100 NMR spectrometer equipped with a Nicolet TT-100 data system. Major peaks are assigned in Table 2. Minor peaks arise from the presence of cis-trans isomerism at the amide bond (16).

Table 1

Proton-NMR Data for Captopril

<u>Proton</u>	<u>Chemical Shift (δ)</u> ¹ PPM from TMS(ext.)
COOH	9.81 (s)
α -CH	4.60 (m)
β -CH ₂	2.03 (m); 2.25 (m)
γ -CH ₂	2.07 (m)
δ -CH ₂	3.63 (m)
$-\text{CH}-\overset{\text{O}}{\underset{\text{ }}{\text{C}}}$	2.44 (d,q) J=6,9
$-\text{S}-\text{CH}_2$	2.82 (m)
$-\text{CH}_3$	1.17 (d) J=6
SH ³	1.53 (dd) J=9,8

¹ multiplicities: d=doublet; q=quartet;
m=multiplet. J=proton-proton coupling
constants in Hertz.

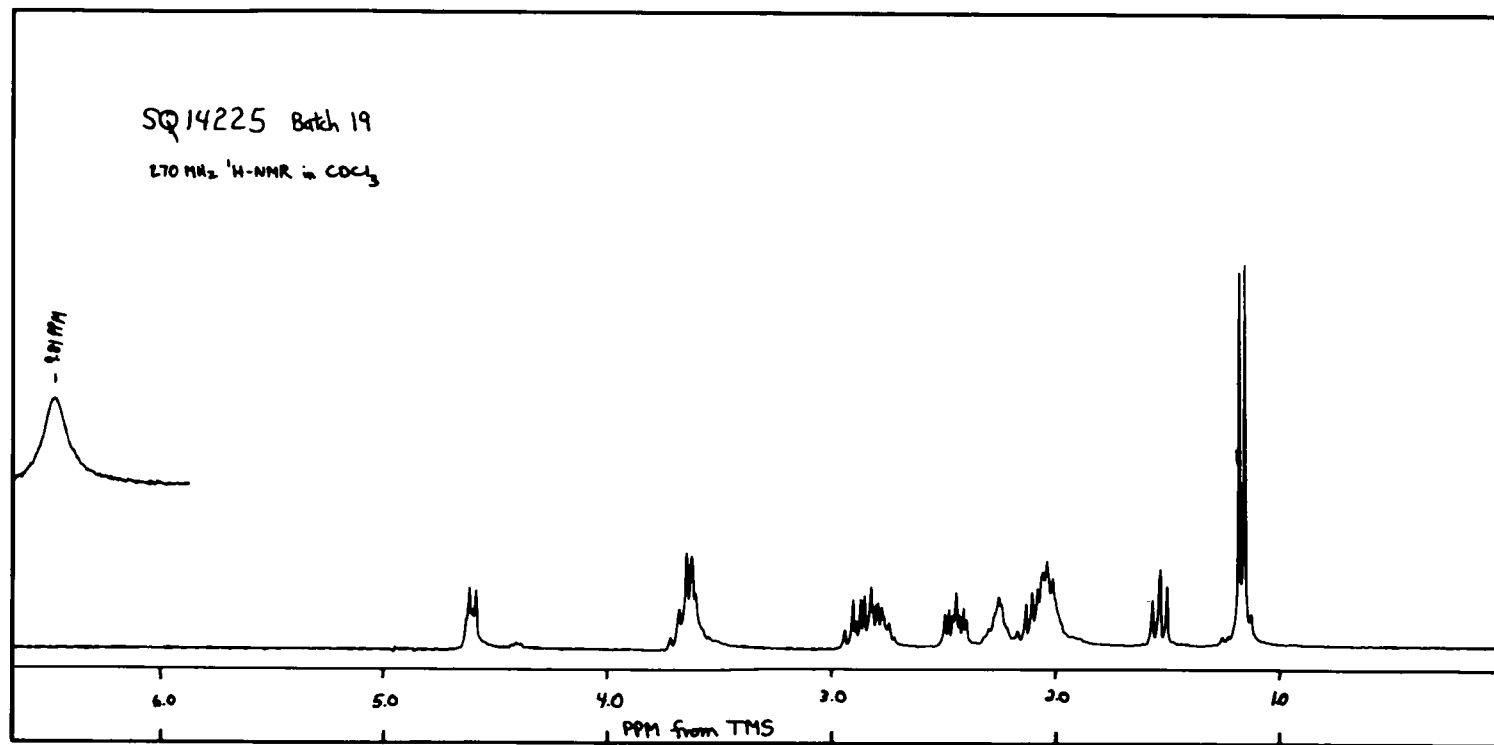


Figure 7. 270 MHz ^1H -NMR Spectrum of Captopril in CDCl_3

Instrument: Bruker HX-270

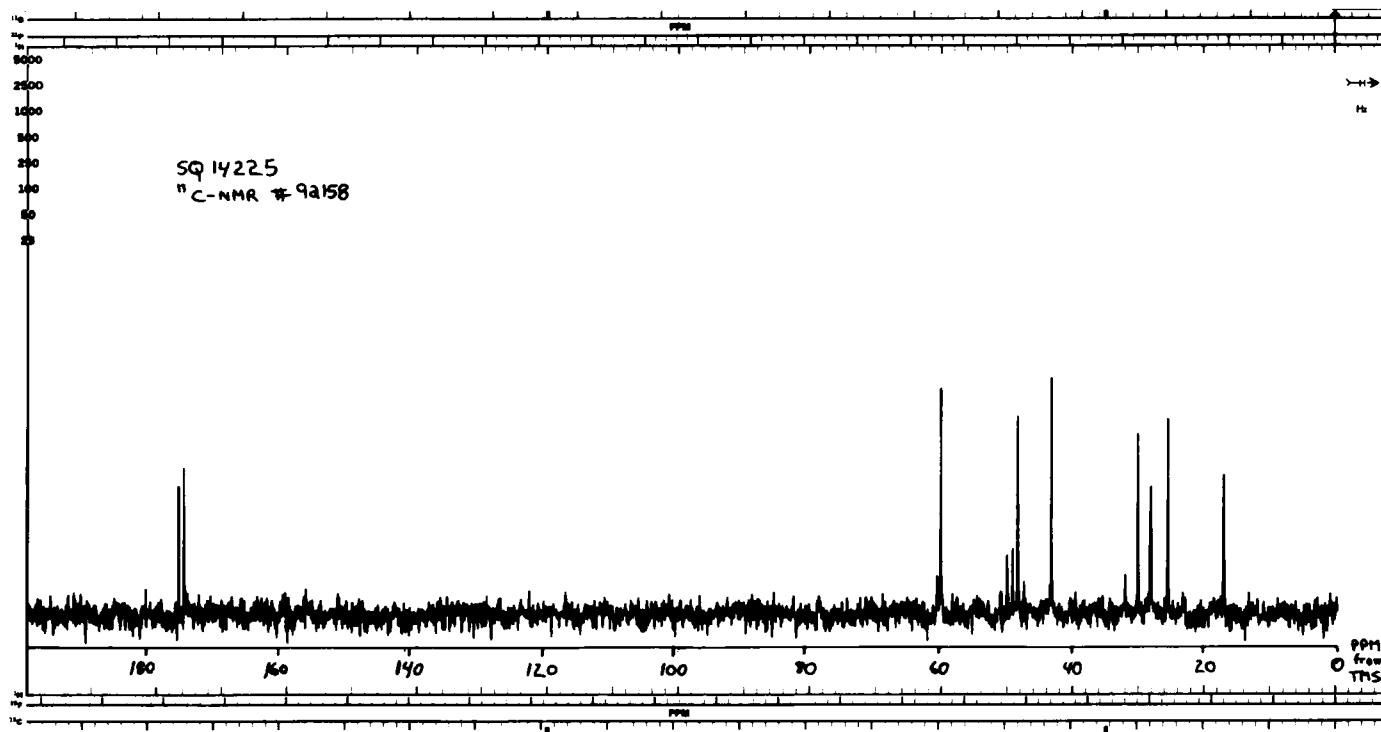


Figure 8. 100 MHz ^{13}C -NMR Spectrum of Captopril in CD_3OD

Instrument: Varian XL-100

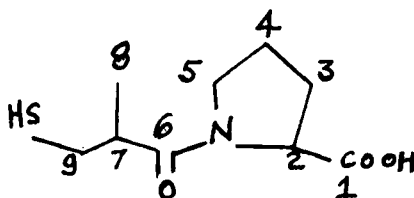


Table 2

Carbon-13 NMR Data for Captopril in CD₃OD.

Carbon #	Chemical Shift (δ) ¹ ppm from TMS
1	175.69
2	59.84
3	30.03
4	25.49
5	48.24
6	174.91
7	43.1
8	17.07
9	28.1

¹ Referenced from center peak of the CD₃OD multiplet at 49.0 ppm

4.13 Ultraviolet Spectra

Spectra of captopril in aqueous methanol, (Fig. 9) water, 0.1M sodium hydroxide and 0.1M hydrochloric acid (Fig. 10), are presented (17). These spectra depict an end absorption slope without peak or shoulder. The slope spectrum in 0.1M sodium hydroxide was shifted considerably towards higher wavelengths. Since weak sulfhydryl absorption is reported (18) in the 220-230 nm region, this absorption shift may be due to ionization of the sulfhydryl function by the alkali. This shift towards higher wavelengths with increase in pH has been used by Ondetti (19) to determine the pK_a of the sulfhydryl in captopril (Section 4.32).

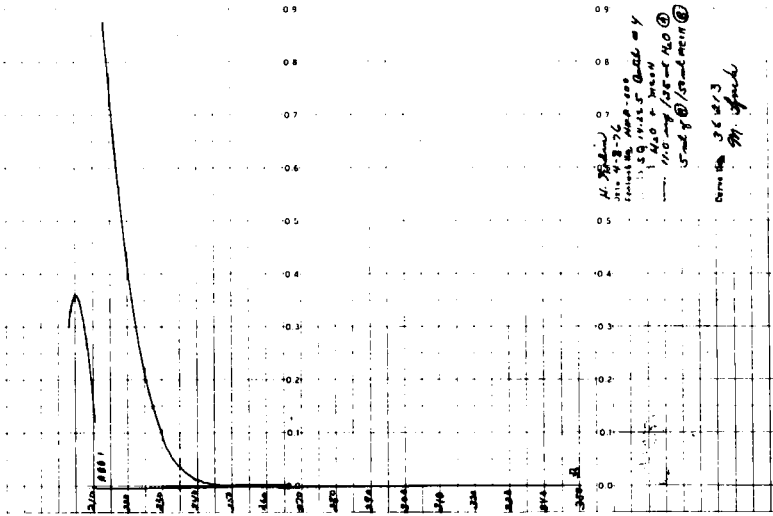


Fig. 9. Ultraviolet absorption spectra of Captopril in 10% aqueous methanol solution.

Instrument: Cary 15.

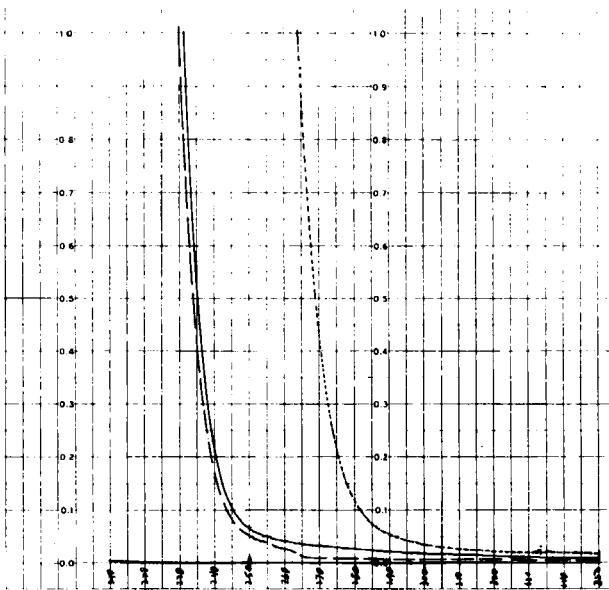


Fig. 10. Ultraviolet absorption spectra of Captopril in H₂O, 0.1N HCl and 0.1N NaOH

Instrument: Cary 15

The spectra suggest that there is a maximum at about 200 nm, attributable to the thiol function. However, precise determination of the peak absorbance was difficult because the extremely large blank absorbances prevented maintenance of a stable balance at this wavelength. Consequently the peak maximum is uncertain in Figure 9. However the peak absorbance is valid in Figure 15 in which the solvent was an HPLC mobile phase (System 4 - Table 8 - Section 6.32).

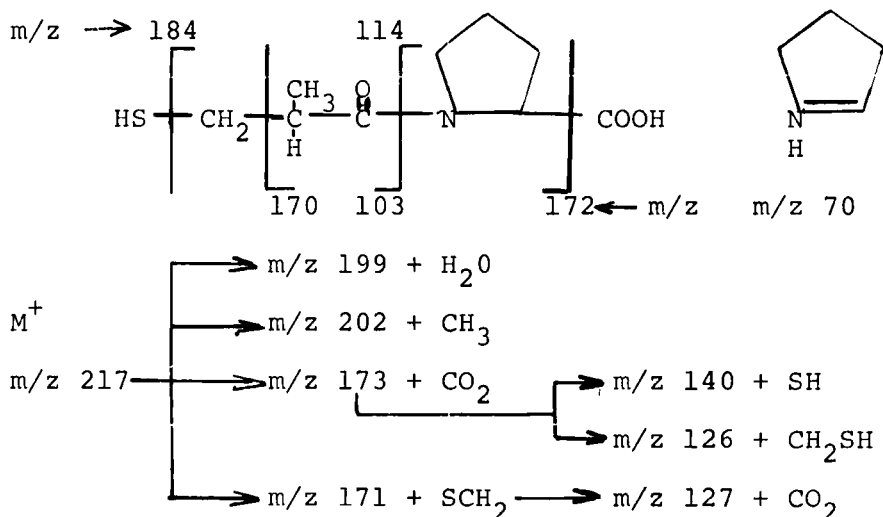
4.14 Mass Spectrum

The mass spectral pattern indicated in Figure 12 was obtained (20) on an AEI MS-9 Mass Spectrometer.

The fragmentation, responsible for the spectrum in Figure 12, is depicted schematically in Figure 11. The M^+ of m/z 217 and the other fragments are consistent with a sulfur-containing compound of the composition $C_9H_{15}NO_3S$ (Section 1.1).

Figure 11

Mass Spectral Fragmentation Schematic Captopril



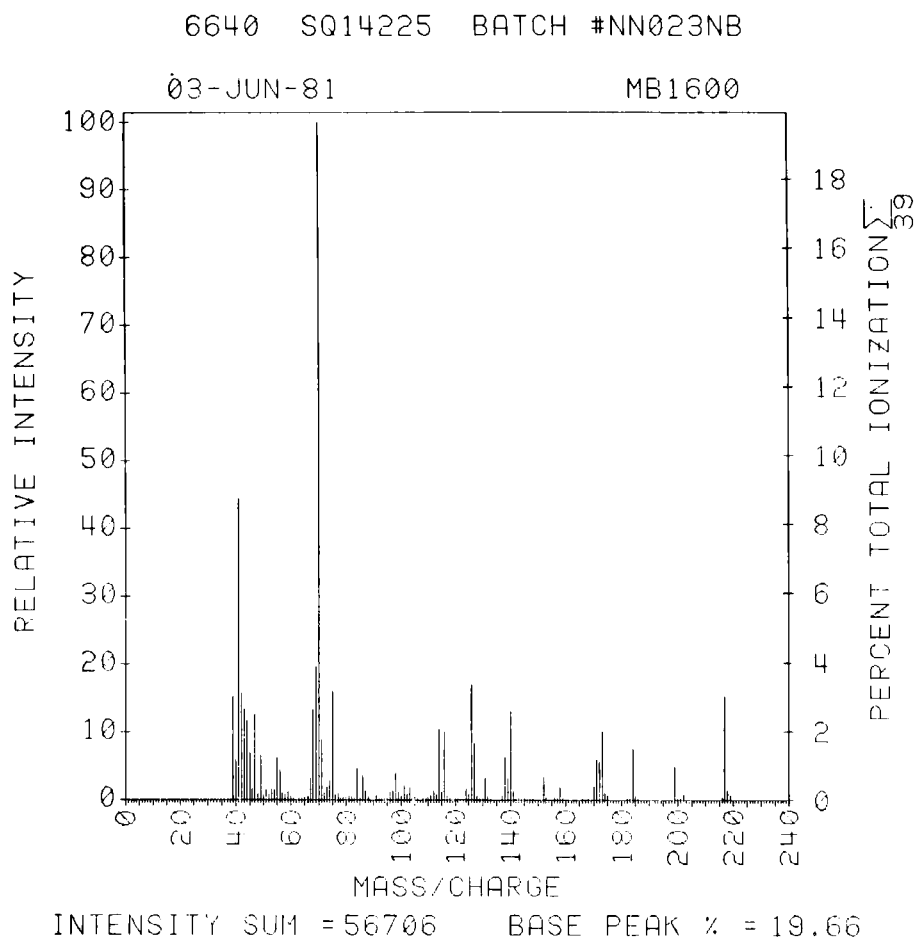


Figure 12. Mass Spectrum of Captopril

Instrument: AEI MS-902 Spectrometer Equipped with
Frequency Modulated Tape Recorder,
Spectrum Processed on Digital
Equipment Corporation PDP-11 Computer

4.2 Solid State Properties

4.21 Polymorphism

An unstable, low (86°C) melting and a stable, high (106°C) melting form of captopril have been observed. These forms exhibited different unit cells (Section 4.26) on single crystal X-ray examination, differences in their powder X-ray (Section 4.27), and differences in the solid state infrared spectra (Figures 5 and 6). Agreement of their optical rotations, infrared in solution and bioassays established them as polymorphs.

4.22 Differential Thermal Analysis (D.T.A.)

DTA of the high melting polymorph (22) yielded a sharp, well-defined endotherm at 106°C whereas the low melting polymorph produced a sharp endotherm at 86°C. When the low melting polymorph was allowed to resolidify and the DTA repeated, the endotherm at 86°C had disappeared and an endotherm at 106°C appeared. The latter suggests that the high melting form is the stable polymorph. A DuPont 900 Thermoanalyzer programmed for a temperature rise of 15° per min was utilized for these thermograms.

4.23 Melting Range

The U.S.P. (Class 1) melting range for the high melting polymorph was 105.2 - 105.9° (21). This agrees well with its D.T.A. endotherm of 106°C. The low melting polymorph has a melting range of 87-88°, in agreement with its DTA endotherm of 86°.

4.24 Differential Scanning Colorimetry (D.S.C.)

Use of DSC as a purity index for captopril is supported by titrimetric assays (17) of the carboxyl function (alkalimetry) and of the sulfhydryl function (iodimetry). For instance for batch 4 these yielded 99.6% for the carboxyl and 99.2% for the sulfhydryl in very good agreement with the DSC of 99.7% mole % (22).

4.25 Hygroscopicity

Under ordinary conditions captopril is not hygroscopic. Equilibrium moisture studies (23) indicate no moisture pickup by captopril up to 50% relative humidity at room temperature. Above 50% R.H. it shows a tendency to cake after one to two days.

Captopril did not exhibit any visual physical changes and remained dry from 0 to 67% R.H. on exposure for 14 days. Samples exposed to 81% R.H. for 14 days appeared moist (24).

4.26 Single Crystal X-ray Diffraction

Single crystal X-ray analyses have been completed (25) for both the low (melting range 86-87°C) and high (melting range 105-106°) melting polymorphs. Both forms are orthorhombic with the following crystal data:

A. High melting polymorph $a = 6.834(2)$, $b =$

$8.821(2)$, $c = 17.982(4)\text{\AA}$; $V = 1084\text{\AA}^3$; space group $P2_12_12_1$ with four molecules per unit cell; calculated density = 1.33 gcm^{-3} . Refined to $R = 0.04$ for the 745 observed single crystal intensities.

B. Low melting polymorph $a = 9.496(3)$, $b =$

$12.304(3)$, $c = 19.282(5)\text{\AA}$; $V = 2253\text{\AA}^3$; space group $P2_12_12_1$ with eight molecules per unit cell; calculated density = 1.28 gcm^{-3} . Refined to $R = 0.06$ for the 1093 observed single crystal intensities.

The structure in both has the S,S absolute configuration with a Z (*Trans*) conformation about the N-C(O) amide bond (the O-C-N-C(2) dihedral angles vary from -4 to +6°). The molecular conformation differ in detail, most notably in the conformation about the (S)C-C(CO) bond.

Atomic coordinates relative to orthogonal axes for the high melting form are:

<u>Atom</u>	<u>X</u>	<u>Y</u>	<u>Z</u>
S	-7.010	1.595	-5.766
N1	-6.499	-1.729	-1.938
C2	-5.799	-2.755	-1.152
C3	-6.274	-2.492	0.282
C4	-7.067	-1.228	0.252
C5	-7.511	-1.024	-1.144
C6	-6.188	-4.132	-1.626
O6A	-7.165	-4.364	-2.286
O6B	-5.394	-5.106	-1.204
C7	-6.215	-1.576	-3.227
O7	-5.308	-2.253	-3.754
C8	-7.041	-0.624	-4.050
C9	-6.163	0.238	-4.947
C10	-8.052	-1.432	-4.821

It was predicted that salt formation with resultant dissociation to a carboxylate anion would influence Capoten to crystallize in its less common E (*cis*) conformation. This prediction was tested (25) by performing single crystal analysis on the dicyclohexylamine salt of captopril. The analysis indicated that the salt was indeed in the E conformation, i.e. the carbonyl groups of the amide and carboxyl functions are *cis* to each other.

4.27 Powder X-ray Diffraction

The stable, higher melting polymorph and the lower melting, metastable polymorph are shown in the powder X-ray patterns Figures 13 and 14 respectively (26). The values given in the patterns are also listed in Tables 3 and 4 for the high and low melters respectively. The tables also show the relative intensities (based on peak areas) of the various peaks.

A powder X-ray pattern taken on the low melting polymorph after it was heated to about 95°C showed conversion to the stable form.

The X-ray pattern was taken with copper K_{α} , nickel filtered X-radiation.

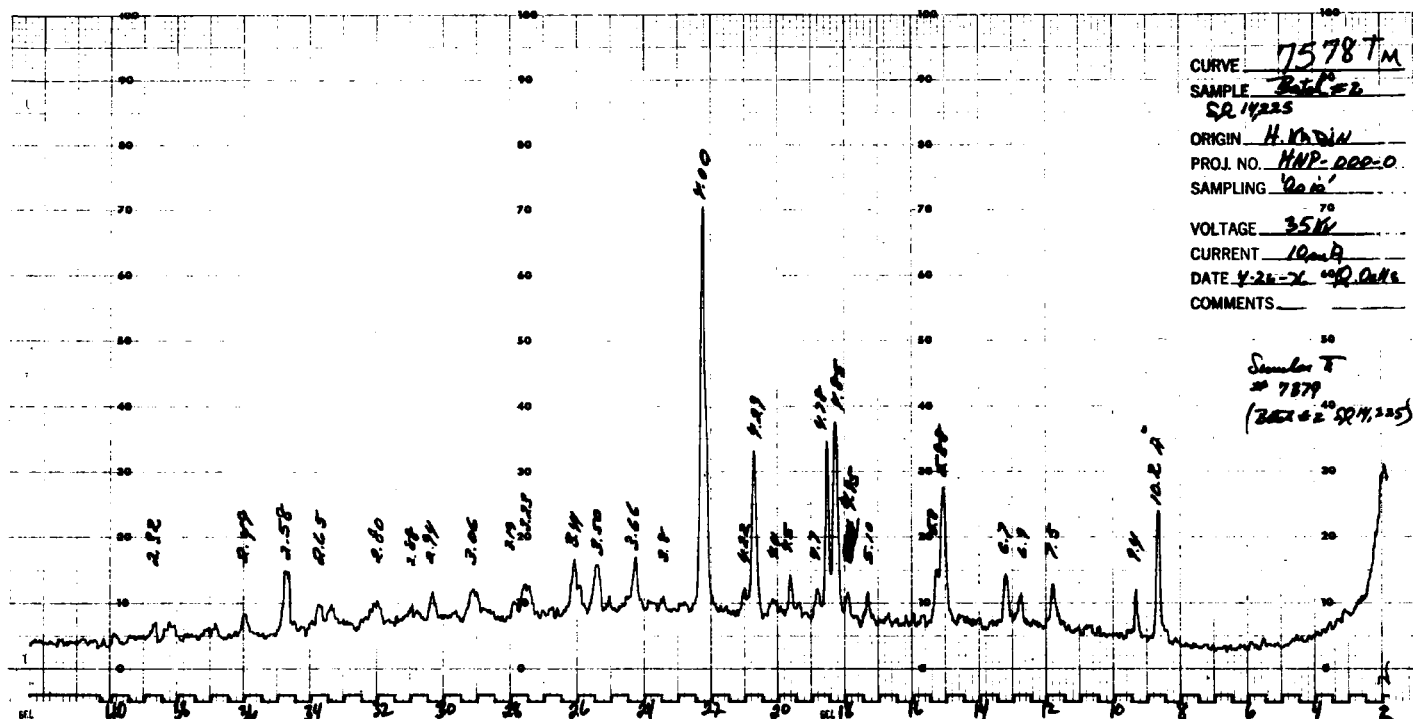


Figure 14. Powder X-Ray Diffraction Curve of Captopril, Low Melting Polymorph

Instrument: Phillips X-Ray Powder Diffractometer

Table 3

Powder X-Ray Diffraction Data for Figure 13 (High Melting Polymorph)

2- θ (DEG.)	D(ANGSTROMS)	PEAK	REL. PEAK	AREA	REL. AREA
9.99	8.85	22.7	0.208	88.1	0.270
11.35	7.80	37.9	0.347	138.4	0.424
14.24	6.22	15.2	0.139	122.3	0.374
16.45	5.39	21.0	0.192	77.5	0.237
17.21	5.15	57.1	0.523	169.6	0.519
17.98	4.93	47.5	0.435	166.8	0.511
19.25	4.61	34.4	0.315	146.3	0.448
19.85	4.47	109.1	1.000	324.5	0.994
20.78	4.27	37.7	0.346	130.1	0.398
22.23	4.00	49.8	0.456	161.7	0.495
24.52	3.63	24.1	0.221	86.4	0.265
25.03	3.56	15.3	0.140	60.2	0.184
25.97	3.43	62.6	0.574	326.6	1.000
26.56	3.36	13.7	0.126	68.2	0.209
28.26	3.16	68.7	0.630	243.1	0.744
29.79	3.00	10.6	0.097	102.0	0.312
30.81	2.90	8.4	0.077	42.5	0.130
31.66	2.83	9.1	0.083	43.9	0.134
33.45	2.68	8.3	0.076	47.9	0.147
34.28	2.61	17.9	0.164	98.2	0.301

Table 3 (Continued)

2- θ (DEG.)	D(ANGSTROMS)	PEAK	REL. PEAK	AREA	REL. AREA
36.17	2.48	14.8	0.136	62.3	0.191
36.34	2.47	13.3	0.122	37.5	0.115
37.53	2.40	9.3	0.085	64.9	0.199
38.63	2.33	15.3	0.140	73.3	0.224

Sorted Data (Highest Peak First)

2- θ (DEG.)	D(ANGSTROMS)	PEAK	REL. PEAK	AREA	REL. AREA
19.85	4.47	109.1	1.000	324.5	0.994
28.26	3.16	68.7	0.630	243.1	0.744
25.97	3.43	62.6	0.574	326.6	1.000
17.21	5.15	57.1	0.523	169.6	0.519
22.23	4.00	49.8	0.456	161.7	0.495
17.98	4.93	47.5	0.435	166.8	0.511
11.35	7.80	37.9	0.347	138.4	0.424
20.78	4.27	37.7	0.346	130.1	0.398
19.25	4.61	34.4	0.315	146.3	0.448
24.52	3.63	24.1	0.221	86.4	0.265
9.99	8.85	22.7	0.208	88.1	0.270

Table 4

Powder X-Ray Diffraction Data for Figure 14 (Low Melting Polymorph)

2- θ (DEG.)	D (ANGSTROMS)	PEAK	REL. PEAK	AREA	REL. AREA
8.71	10.15	24.5	0.392	68.7	0.259
9.31	9.50	13.7	0.219	26.3	0.099
11.77	7.52	14.3	0.229	89.1	0.336
12.71	6.96	14.0	0.224	43.0	0.162
13.13	6.74	16.6	0.266	69.4	0.261
15.00	5.91	31.8	0.509	142.4	0.536
17.21	5.15	14.7	0.235	43.2	0.163
17.89	4.96	14.0	0.224	36.8	0.138
18.23	4.87	39.5	0.632	101.0	0.380
18.40	4.82	34.8	0.557	77.2	0.291
19.51	4.55	16.9	0.270	91.2	0.343
20.10	4.42	13.1	0.210	57.3	0.216
20.61	4.31	30.6	0.490	112.0	0.422
20.87	4.26	13.1	0.210	34.8	0.131
21.38	4.16	13.0	0.208	33.8	0.127
22.14	4.02	62.5	1.000	265.5	1.000
23.25	3.83	13.9	0.222	69.1	0.260
23.50	3.79	12.1	0.194	34.4	0.130
24.10	3.69	16.8	0.269	113.4	0.427
24.86	3.58	12.5	0.200	43.4	0.163
25.20	3.53	17.4	0.278	83.3	0.314

Table 4 (Continued)

2- θ (DEG.)	D (ANGSTROMS)	PEAK	REL. PEAK	AREA	REL. AREA
25.88	3.44	17.8	0.285	105.0	0.395
27.33	3.26	14.6	0.234	90.5	0.341
28.35	3.15	12.8	0.205	33.3	0.125
28.94	3.09	14.4	0.230	104.1	0.392
30.05	2.97	14.0	0.224	72.8	0.274
34.47	2.60	16.6	0.266	99.3	0.374

Sorted data (Highest Peak First)

2- θ (DEG.)	D (ANGSTROMS)	PEAK	REL. PEAK	AREA	REL. AREA
22.14	4.02	62.5	1.000	265.5	1.000
18.23	4.87	39.5	0.632	101.0	0.380
18.40	4.82	34.8	0.557	77.2	0.291
15.00	5.91	31.8	0.509	142.4	0.536
20.61	4.31	30.6	0.490	112.0	0.422
8.71	10.15	24.5	0.392	68.7	0.259
25.88	3.44	17.8	0.285	105.0	0.395
25.20	3.53	17.4	0.278	83.3	0.314
19.51	4.55	16.9	0.270	91.2	0.343
24.10	3.69	16.8	0.269	113.4	0.427
34.47	2.60	16.6	0.266	99.3	0.374
13.13	6.74	16.6	0.266	69.4	0.261
17.21	5.15	14.7	0.235	43.2	0.163

4.3 Solution Data

4.31 Solubility

Captopril at 25°C is freely soluble (1 to 10 parts solvent to 1 part solute) in water, methanol, ethanol (SD3A), isopropanol, chloroform, or methylene chloride. However, it is only soluble (10-30 parts solvent to 1 part solute) in ethyl acetate (27). The solubility of captopril in water, at 25°C, is 160 mg/ml (28). A solubility-temperature profile of captopril in water obeyed a linear equation up to 40°C (28). Beyond this temperature captopril showed extraordinarily high water solubility.

Solubility in sesame and corn oils was less than 1 mg/ml at 25°C, whereas the solubility in the synthetic oil triacetin (glyceryl triacetate), at 25°C, was greater than 20 mg/ml (29).

4.32 pK_a

The pK_a of the carboxyl of captopril (pK₁) is reported (29) to be 3.7. Whereas a carboxyl break was readily observed with alkali potentiometry, the sulfhydryl break could not be detected (17). Therefore, the pK_a of the sulfhydryl in captopril (pK₂) was not estimated by classical potentiometry. It was, however, estimated at 9.8 (pK₂) by Ondetti (19) and Weiss (30) using sulfhydryl u.v. shifts to higher wavelengths with increase in pH (Section 4.13). The method utilized was adapted from Benesch and Benesch (31).

4.33 Metal Complex Formation

Captopril was modelled (11) as a selective and competitive inhibitor of the angiotensin converting enzyme (Section 2). Part of this inhibition resides in the binding of the zinc cofactor within the enzyme's active site by captopril's thiol function. Constrained within the active site by the multiple interactions of site and inhibitor, captopril bars entry of angiotensin I and thus prevents its conversion to the most powerful natural pressor, angiotensin II. Since captopril lacks an amino group, it does not

complex metals in solution with the well-documented avidity (32,33) of amino group bearing thiols like cysteine, glutathione, and *in vivo* metal depletors like 2-mercaptopropionyl glycine and penicillamine. Indeed, Weiss (30) reported that an alkali potentiometric study of the extent of zinc ion complexation with cysteine (I), 2-mercaptopropionyl glycine (II), and captopril (III) indicates the order of binding, at pH 7.4, to be I > II > III.

Captopril binds mercuric ion (Section 7) to block its colorimetric reaction with Ellman's reagent, thus allowing a measurement of non-sulphydryl colorimetric interferences.

4.34 Optical Rotation

The optical rotation of the captopril in absolute ethanol (34), using the Perkin-Elmer 141 Automatic Polarimeter, was determined to be: $\alpha_D^{25} = -127.8^\circ$. The R,S isomer rotates at about $+5^\circ$.

4.35 Partition Coefficients

A partition ratio (solvent/aqueous) after shaking equal volumes of cosaturated aqueous (pH 2) and methylene chloride was 1.39 (21). A comparably determined partition ratio between equal volumes of cosaturated 0.1M HCl and octanol was 1.9 (30). When utilizing salting out partition from aqueous acid into methylene chloride at the captopril concentrations prevalent in the urine analysis (about 25-50 mcg/ml for a 100 mg dose) NaCl but not Na₂SO₄ was found to enhance captopril oxidation (Section 7). This has been attributed to trace chlorine generation from acidic chloride plus oxygen.

5. Stability

5.1 Solid State Stability

No significant decomposition was detected (35) in SQ 14,225 bulk samples, stored at $+5^\circ\text{C}$, $+33^\circ\text{C}$ and $+50^\circ\text{C}$ for up to 6 months or exposed to 900 foot-candles in a light box for 30 days, when compared to -20°C samples which served as the

control. Samples were examined for appearance, color, odor, LD₅₀ safety and by quantitative TLC and HPLC, iodimetric titration, infrared, and optical rotation.

5.2 Solution Stability

Captopril in aqueous solution undergoes an oxygen facilitated, first order, free radical oxidation at its thiol to yield captopril disulfide (28). Hydrolysis at the amide linkage occurs only under forcing conditions (see Section 5.25). Oxidation was delayed by adjustment to lower pH, addition of chelating agents, increasing captopril concentration, utilization of nitrogen or low oxygen headspace, and incorporation of antioxidants. Oxidation seems to occur less readily in methanol (36). No degradation of captopril was observed (40 mcg/ml) in this solvent for up to 2 weeks at 5°C.

5.21 Stability and Solution pH

Oxidation rate constants at various pH values (28) in Table 5, suggest that captopril is optimally stable below pH 3.5, the oxidation rate being essentially constant from pH 2 to 3. The rate constants increase rapidly above pH 4. Using HPLC and colorimetry (38), captopril aqueous stability was studied, at 50 mcg/ml, in a rotating basket dissolution apparatus for up to 180 minutes at 37°C in distilled water, and at pH 1, 2 and 3. Excellent stability at pH 1 and 2 but appreciable degradation at pH 3, and in distilled water was observed. Surprisingly, the rate of degradation at pH 3 exceeded that in distilled water. The more rapid oxidation at pH 3 was attributed to catalysis via greater trace metal solution from the dissolution baskets.

Table 5

Oxidation Rate Constant for Captopril (5 mg/ml)
in Citrate-Phosphate Buffers at Various pH
Values at 50°C

pH	Rate Constant (day ⁻¹) x 10 ³
2.13	8.38
2.59	9.01
2.89	8.22
3.13	8.31
3.53	9.92
3.88	9.13
4.23	12.94
4.67	19.43
5.16	28.93
5.63	42.03

5.22 Solution Stability, Metal Ions and Chelating Agents

Transition metal ions most effectively catalyze oxidation of captopril through a recycling of oxygen free radicals (28). The most effective of these catalysts are ubiquitous copper and iron, in given order. As little as 1 ppm of copper has been observed to catalyze captopril oxidation in solution (28).

As has been demonstrated with cysteine (39) lower levels of disodium edetate (EDTA Na₂) may enhance metal ion catalyzed thiol oxidation, whereas higher levels retard oxidation.

Disodium edetate 0.1% (Na₂EDTA 0.1%) best stabilized 0.5 mg captopril per ml (of citrate-phosphate buffer at pH 4, μ = 0.5) in Teflon-faced rubber sealed vials (37).

Analysis of urinary captopril was necessary for dosage form bioavailability and dose titration studies. The necessity for long term storage of samples prior to analysis required development of an acid-chelate stabilization (40). This stabilization utilized diethylenetriamine pentaacetic acid (DTPA) reputed (40) to be a more effective metal chelator than Na₂EDTA. A

relatively large amount of DTPA (about 400 mg in 5 ml) was mixed into the periodic urine voiding. This was followed by an acidification to about pH 2 with a mixture of citric and oxalic acids and a rapid refrigeration. DTPA, citric and oxalic have been reported (41) to be effective sequestering agents in the pH range 2 to 5 for Al, Cu, Fe, Ni and Zn. Analysis of captopril in urine samples stabilized in this manner, before and after 90-120 days of refrigeration, agreed remarkably well (40).

5.23 Concentration and Solution Stability

The greater the captopril concentration, the slower the oxidation (28). For example, no significant degradation (within $\pm 3\%$) could be detected by automated Ellman colorimetry (42) at a concentration of 250 mg captopril per ml of solution at pH 12.5-14 after overnight room temperature storage in uncovered beakers. At the opposite extreme, captopril at an analysis concentration of 25-50 micrograms per ml of solution at pH 13.5 stored overnight at room temperature in open tubes lost 84% of its sulphydryl activity (21).

5.24 Oxygen Tension and Solution Stability

The following 2 1/2 hour accelerated stability at 40°C on 25 ml of 1 mg captopril per ml solutions in unstoppered 100 ml volumetric flasks using air, oxygen, or nitrogen purging, where indicated, was carried out (28).

Table 6

Oxygen Tension and Solution Stability

Code	M <u>Na₂HPO₄</u>	pH	ppm <u>Cu</u>	<u>Purge</u>	% Captopril <u>Recovered (HPLC)</u>
1	0.08	7.9	2	None	73
2	0.08	7.9	2	Air	58
3	0.16	7.5	10	None	88
4	0.16	7.5	10	Air	62
5	0.08	7.8	2	N ₂	96
6	0.04 (KH ₂ PO ₄)	7.8	1	O ₂	0

Under these conditions it is apparent that oxygen causes rapid, complete degradation, air facilitates degradation, and nitrogen protects captopril in solution.

5.25 Amide Hydrolysis and Solution Stability

Captopril solutions at 5 mg per ml in 0.5 M HCl, containing 0.1 mg EDTA Na_2 /ml to minimize oxidation, were heated at elevated temperatures. The rate of hydrolysis was monitored by HPLC. The data yielded first order linear plots from which Wang (43) calculated rate constants (Table 7). An Arrhenius plot yielded a heat of activation for amide hydrolysis of 21.4 kcal/mole, comparable to other amides.

Table 7

Rate Constants for Captopril Hydrolysis

		<u>k</u>	<u>t_{90%}</u>
40°C	pH 3	$5.5 \times 10^{-7} \text{ hr}^{-1}$	10 years
40°C	pH 4	$5.5 \times 10^{-8} \text{ hr}^{-1}$	10 years
22°C	pH 3	$6.7 \times 10^{-9} \text{ hr}^{-1}$	86 years
22°C	pH 4	$6.7 \times 10^{-10} \text{ hr}^{-1}$	86 years

It is clear from these $t_{90\%}$'s (times for 90% amide hydrolysis), that hydrolysis contributes insignificantly to degradation.

6. Analytical Tests and Methods

6.1 Elemental Analysis

The following results were obtained:

Element Calculated		Found for Batch 5
C	49.78	49.97
H	6.96	6.84
N	6.45	6.50
S	14.77	14.63

6.2 Spectrophotometric Methods

6.21 Colorimetry

Spectrophotometry for captopril has included the widely utilized Ellman's reaction (44) in which sulfhydryl reduction of 5,5'-dithiobis-2-nitrobenzoic acid yields a mole of intensely yellow 2-nitro-5-thiobenzoate anion per mole of captopril. Manual (40,45) and automated colorimetry have been utilized for captopril analysis in chows (Section 7.71), urine (Section 7.72) and in pharmaceutical formulations by TLC (Section 6.31).

Kinetic colorimetry vs pH (from pH 5 to 10) studies (46) of a manual version of Ellman's colorimetry of captopril established that the pH optimum was at pH 7 (1 M phosphate, 0.05 M EDTA). Maximum color was attained within 2 min. and stability was maintained for at least 45 min.

The S-nitroso-Bratton-Marshall colorimetry (47) has been applied to analysis for captopril in various formulations (48). It was adapted from a method (47) for cysteine in biological materials. In this method the thiol reacts with nitrous acid to form a relatively stable S-nitroso derivative. Excess nitrous acid is destroyed by sulfamic acid. The S-nitroso derivative is then hydrolyzed by mercuric ions to release nitrous acid. The latter diazotizes sulfanilamide, presumably at a faster rate than destruction of the nitrous acid by

excess sulfamic acid. The diazotized sulfanilamide is then coupled to N-(1-naphthyl)ethylenediamine to yield a stable measureable red azo dye. The method was found to be less suitable for analysis of captopril in urine than Ellman's Reaction (Section 7.72).

Five simple, spectrophotometric identity tests for captopril reported by Valatin (49) include the observation of an evanescent red when captopril reacts with nitrous acid. In addition captopril color tests were described yielding purple with nitroprusside, blue with ferric chloride, red on addition of neutral N-ethylmaleimide followed by strong alkali, and finally orange-yellow (specific for proline) on acid hydrolysis followed by neutralization then reaction with ninhydrin.

6.22 Fluorometry

Captopril was reacted with N-[p-(2-benzoxazolyl)-phenyl] maleimide in pH 6.85 buffer and the fluorescence of the captopril-maleimide adduct was then measured at 310 nm excitation and 365 nm emission (49).

For coupling of captopril to N-(7-dimethylamino-4-methyl coumarinyl) maleimide to yield a highly fluorescent derivative see Section 7.5.

6.3 Chromatographic Methods

6.31 Thin Layer Chromatography (TLC)

For quantitation of captopril (purity assay) standard and sample solutions are each chromatographed at 100 μ g and 200 μ g levels on Analtech silica gel G plates using the solvent system benzene-acetic acid (75:25). The captopril zones on the plate are located by spraying a guide-channel with a basic methanolic solution of 5,5'-dithiobis-2-nitrobenzoic acid. Captopril on the guide-channel appears as a yellow zone. The silica gel containing captopril zones on the untreated portion of the plate are removed from the plate, eluted with 5% aqueous trichloroacetic acid, and reacted with a methanolic solution of 5,5'-dithiobis-2-nitrobenzoic acid, at an alkaline

pH, to form an intensely yellow 2-nitro-5-thiobenzoate anion that is measured at 412 nm in a spectrophotometer (50).

In a thin-layer semi-quantitative procedure for measuring the individual impurities in captopril, samples are chromatographed at 100 μ g and 200 μ g levels and standards are chromatographed at concentrations ranging from 0.5 μ g to 4.5 μ g. TLC separation is carried out on Analtech Silica Gel G plates, developed in conventional and in continuous development chambers, using the solvent system benzene-acetic acid (75:25). After development, the plates are air dried and placed in a chamber saturated with iodine vapors. A semi-quantitative estimate of the concentration of each impurity in the sample is made based on a visual comparison of the size and intensity of each impurity zone with the appropriate standard zones (50).

These TLC procedures, after appropriate initial extractions, have been adapted for formulations. They have also been applied to semi-quantitative analysis of captopril disulfide in tablets and as a TLC identity test for captopril in tablets (50).

Streaked or spotted plates should be introduced into the developing chamber (2 per chamber) immediately after applying the solutions to the plates. This minimizes the conversion of captopril to its disulfide.

Placebo powders were spiked with known amounts of captopril and assayed using the TLC procedure. The results of 15 assays gave a recovery of 100.6%, a standard deviation (s) of 1.39 and a coefficient of variation (C.V.) of 1.38 (50).

6.32 High-Performance Liquid Chromatography (HPLC)

Three HPLC systems were investigated (51) for selective separation of captopril from pharmaceutical excipients, synthetic intermediates, degradation products and impurities. These included anion exchange, amino, and

octadecylsilane (ODS) systems. The first and second appeared not to be the systems of choice when they did not achieve necessary separations of pharmaceutical excipients from captopril. A heavily loaded version of the third system (15% ODS, monolayer bonded on silica, Partisil ODS-2) was selected as optimum for bulk and tablet analysis. The third system was clearly superior. Nevertheless, the first two systems had very occasional usage when formulations contained excipients which interfered with captopril or its disulfide analysis in the heavily loaded Cl8 system.

The characteristics of these systems and of several others investigated are summarized in Table 8 below.

Since captopril has a U.V. absorption maximum at about 200 nm with broad end absorption (see Figure 15) U.V. detection was at 230 nm or lower, compatible with a good baseline and low solvent interference inherent in a good signal to noise ratio. An alternative to U.V. detection, for captopril per se (see Section 7.4 for captopril u.v. derivative HPLC) is the electrochemical detector (systems 8 and 9) introduced for sulfhydryl analysis by Saetre and Rabenstein (52). The electrochemical detector's near neutral detection potential makes it especially thiol selective. It has been reported (53) to have a sensitivity at least 200 fold over that of U.V. detection.

Table 8

Captopril HPLC Systems

System No.	Flow Rate ml/min	Loop Injection Volume μ l	Stationary Phase	Mobile Phase	Detection	Remarks and Reference
1	0.6	20	250 x 4.6 mm Partisil SAX 10 μ m Strong anion Whatman	525 mg citric acid. H ₂ O +37.7mg ² NH ₄ CIT to 1 liter with CH ₃ OH adjust to pH ³ 3.30 with 0.1 N HCl	U.V. 220 nm U.V. 220 nm	51 51
2	1.0	20	300 x 3.9 mm μ Bondapak NH ₂ 10 μ m amino Waters	0.01 M Na ₂ EDTA in 0.05% HAc- CH ₃ CN, 95:5	U.V. 220 nm	51
3	1.0	20	250 x 4.6 mm Partisil ODS 5% 10 μ m ODS mono- layer Whatman	CH ₃ OH-H ₂ O-85% H ₃ PO ₄ ² , 27:75: 0.1	U.V. 220 nm	51

System No.	Flow Rate ml/min	Loop Injection Volume μ l	Stationary Phase	Mobile Phase	Detection	Remarks and Reference
4	1.0	20	250 x 4.6 mm Partisil ODS 15% 10 μ m ODS mono layer Whatman	CH ₃ OH-H ₂ O-85% H ₃ PO ₄ , 50:50:0.05	U.V. 220 nm	51 Optimum resolution system
5	1.0	20	200 mm Hypersil ODS + TSIM 5 μ m ODS + trimethylsilylimidazole monolayers Shandon	CH ₃ OH-H ₂ O-1% H ₃ PO ₄ , 47.5:52.5:1.0	U.V. 230 nm	54
6	1.0	1.0 (ml)	200 mm Hypersil ODS + TSIM 5 μ m ODS + trimethylsilylimidazole monolayers Shandon	CH ₃ OH-H ₂ O-1% H ₃ PO ₄ , 43:57:1.0	U.V. 230 nm	54 Assay disulfide in low potency captopril formulations
7	1.0	20	250 x 4.6 mm Partisil ODS 5% 10 μ m ODS mono layer Whatman	CH ₃ OH-(0.1 M KH ₂ PO ₄ + 15 mM H ₃ PO ₄), 55%:45%	Electrochem., + 0.10 V, Hg Pool vs Ag/AgCl	53

8	0.5	20	300 x 4.6 mm MC ₁₈ Chromegabond 20% 10µm heavily loaded ODS ES Industries	CH ₃ OH-(0.05 M KH ₂ PO ₄ + 30mM H ₂ PO ₄), 55:45%	Electrochem., 55 + 0.10V, Hg film vs. Ag/AgCl	Adapted for "total" urinary captopril analysis
9	1.0	20	250 x 4.6 mm Partisil ODS 5% 10µm ODS Whatman	CH ₃ OH-H ₂ O-85% H ₃ PO ₄ , 20:80: 0.1	U.V. 214 nm	56
10	1-2	Waters Variable Volume	300 x 3.9 mm µ Porasil 10µm silica gel nor- mal phase Waters	CH ₂ Cl ₂ : HAc, 9 : 1 ²	U.V. 240 nm	57 Qual. Sepn. of captopril from its R,S isomer
11	0.5-1	Waters Variable Volume	250 x 4.6 mm Lichrosorb RP18 5µm "capped" ODS Merck	H ₂ O(to pH 3.0 0.5 M H ₃ PO ₄) - CH ₃ CN, 65 : 35	U.V. 220 nm	58
12	100	--	30 x 5.7 cm Prep- PAK C18 ODS Waters	CH ₃ OH-H ₂ O-0.05 M H ₃ PO ₄ , 45 : 55 : 0.05	RI	59 Prep. Sepn. captopril impurities

ODS = Octadecylsilyl

6.33 Gas Liquid Chromatography (GLC)

A captopril- ^{14}C synthesis separated captopril from its R,S optical isomer by GLC of its methyl ester (after CH_2N_2 treatment) on 1.5% OV-17 at 180°C (60). The gas chromatogram of the pre-resolution isomer mixture showed two peaks of almost the same peak area. After a dicyclohexylamine resolution, GLC of the desired captopril (S,S fraction) indicated a single peak at the retention time of the second peak in the chromatogram of the mixture. The method is recommended, over the more conventional, appreciably less sensitive, measurement of optical rotations (Section 4.34), for process control of the isomer separation.

G.C.-mass spectrometry (Section 7.2) and G.C.-flame photometry (Section 7.3) of the N-ethyl maleimide adduct (Section 7) of captopril as methyl and heptafluoroisopropyl esters, respectively, have also been reported.

6.4 Titrimetric Methods

Acid iodate (Iodine) titration, before and after chromatography through a Jones Reductor Column was used to measure disulfide in captopril (61). For small amounts of disulfide this technique measures, relatively inaccurately, small differences between two large numbers. Therefore the subsequently developed, more accurate TLC (Section 6.31) and HPLC (Section 6.32) direct assays are preferred. However the iodine titration, with starch indicator, is useful as a thiol purity assay (61). A "Dead Stop" end point indicator (62) makes the titration more amenable to automation.

7. Analysis in Biological Fluids and Tissues and in Animal Rations

Extreme instability of captopril in biological media necessitated development (63) of an immediate, quantitative thiol derivatization with N-ethylmaleimide (NEM) followed by freezing. Studies (64) with radioactive captopril in whole blood had indicated that a 5 min delay before addition of NEM resulted in a 10% loss of

captopril whereas a 30 min delay yielded a 65% loss. The NEM derivative was found (65) to be stable in frozen whole blood or in blood stored at 5°C for at least 3 months; it was unstable in a frozen or 5°C phosphate buffer (pH 6.85) when stored for more than 4 weeks.

Antioxidants and metal chelators were ineffective (63) as captopril stabilizers in whole blood.

Long term stabilization, at the relatively high concentrations of 1 to 100 mcg per ml prevalent in urine after captopril dosage, has been achieved (40) with metal chelators, acid pH, and 5°C storage (see Section 5.22).

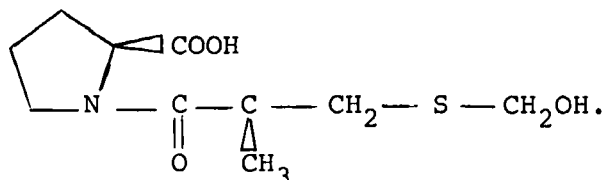
Stability and homogeneity analysis of captopril in animal feeds was required for multiple long range toxicological studies. Despite a high proportion of thiol reactive air, inorganics, and proteins in the feeds, stabilization during the analysis was achieved (46) by an isopropanolic extractant fortified with trichloroacetic acid and metal chelators (see Section 7.71).

7.1 Thin Layer Radiochromatography (TLRC)

NEM stabilization allowed TLRC of captopril in whole blood (63). Aliquots of whole blood were analyzed for total radioactivity and NEM- treated aliquots were extracted with methanol. Reconstituted residues of the extracts were applied to silica gel GF plates, developed with chloroform/ethyl acetate/glacial acetic acid (4:5:3) and analyzed for radioactivity associated with captopril and its disulfide by zonal analysis. The limit of detection was about 10 ng captopril/ml of blood.

In TLRC analysis of the highly radioactive captopril preparations used for drug metabolism studies the thiol of captopril is particularly susceptible to free radical degradation (Section 4.2). Reaction of thiol across the highly active carbonyl double bond of formaldehyde (incorporated in the mobile phase) protects the radioactive captopril during the chromatography (66).

When dilute aqueous solutions of captopril and an excess of formaldehyde are used the product of the reaction has been shown by NMR and IR to be the captopril hemithioacetal.



The hemithioacetal can be reverted to captopril, for sulfhydryl analysis, by acid, base, or bisulfite.

7.2 Gas Chromatography-Mass Spectroscopy (GC-MS)

NEM-Captopril has been determined by GC-MS in the selected ion mode (67) in whole blood, urine (68), amniotic fluid (69), milk (69), and tissues (68) (liver, kidney, lung, and placenta). For determination in whole blood, proteins are precipitated from vortexed whole blood plus internal standard with freshly prepared 10% metaphosphoric acid. The NEM-captopril is absorbed from 0.45 μ M membrane filtrates on purified suction-dried Amberlite XAD-2 resin and eluted with a freshly purified (neutral alumina chromatography) ethyl acetate. The drug is extracted from the ethyl acetate into 5% sodium bicarbonate. After acidification and saturation of the aqueous layer with sodium chloride, the NEM derivative is extracted with ethyl acetate. After evaporation of the solvent extract, samples are methylated for GC-MS.

The G.C. was carried out with dried helium gas at 6 ml/min on a 3% OV-101 column with injector at 280°C and column temperature programming at 200-280°C at 20°C/min. All glass surfaces had been previously silanized with Sylon-CT. The spectrometer was a modified Electronic Associates Quad 300 quadrupole. The samples (1 μ l) were coinjected with 0.5 μ l of N,O-bis(trimethylsilyl) trifluoroacetamide. The latter was used to prolong column life. The peak height intensity

data of the m/z 230 and 248 ions was collected by selected ion monitoring. The limit of detection and the practical detection limit with a 90% confidence limit are 5.5 and 16.5 ng per ml of blood, respectively.

The internal standard, an NEM blocked, deuterated or fluorinated derivative of captopril, compensates for extraction and other possible losses.

The procedure described (67) could be applied to most of the biological media mentioned with but slight revision. However milk required a greater modification. The high fat content of milk made XAD-2 chromatography unfeasible. Therefore NEM-captopril was instead extracted from sodium chloride saturated milk samples with ethyl acetate.

7.3 Gas Chromatography-Flame Photometric Detection (GC-FPD)

A GC method for captopril in blood and urine utilizing a sulfur selective dedicated FPD has been reported (70). Blood is treated with a soln. of N-ethylmaleimide in phosphate buffer soln (pH 7.4) and with metaphosphoric acid (10% soln); after centrifugation, the supernatant soln. is extracted with ethyl acetate, and, after further purification, the extract is evaporated, the captopril-N-hexylmaleimide adduct is added as internal standard, and the mixture is treated to convert the two adducts into their hexafluoroisopropyl esters for analysis by g.l.c. at 215° on a glass column (1 m x 3 mm) packed with 2% of OV-210 on Gas-Chrom Q (80 to 100 mesh), with N (50 ml min⁻¹) as carrier gas and flame-photometric detection. Urine is analysed similarly, but without deproteinisation. The calibration graph (peak-height ratio vs. concn.) is rectilinear for 1 to 5 µg ml⁻¹ of I.

The sensitivity though not specified appears to be in the microgram range. It is therefore not satisfactory for human blood studies which are in the nanogram range.

7.4 High Performance Liquid Chromatography with U.V. Detection (HPLC-UV)

An HPLC procedure for captopril in whole blood and urine has been reported (71). Captopril is thiol-blocked with p-bromophenacyl bromide (pBPAB) or N-(4-dimethylamino-3,5-dinitrophenyl) maleimide (DDPM), then the addition products were separated and determined by HPLC on a reversed phase column. Captopril in blood or urine could be derivatized with pBPAB then excess pBPAB removed by hexane extraction. The captopril disulfides in the same samples were then reduced by tributylphosphine to captopril which in turn were thiol-blocked with DDPM. HPLC of the adduct mixture thus allowed a separate analysis of captopril or disulfides, before and after reduction. The method is reported to be sensitive, and precise down to 5 ng per ml of whole blood and 0.1 mcg per ml of urine.

7.5 Spectrofluorometry

The drug, stabilized with dithioerythritol is absorbed on a Brinkmann XAD-2 column from an acidified diluted blood and eluted with pH 6.9 phosphate-30% dimethylformamide buffer. Captopril in the eluate is reacted with N-(7-dimethylamino-4-methyl coumarinyl) maleimide (DACM) to form a fluorescent derivative (Section 6.22). After acidification, the derivative is extracted into toluene. The fluorescence is measured at 375 nm/425 nm-excitation/fluorescence. The analysis was validated by the GC-MS procedure described above in the range of 0.5 mcg to 10 mcg per ml of blood (72).

7.6 Radioimmunoassay (RIA)

Development and application of a simple, highly sensitive RIA for NEM-captopril in blood plasma has been described (89). The lower detection limit is 2 ng per ml of plasma.

7.7 Semiautomated Ellman Colorimetry, (Figure 16)

7.71 Semiautomated Sulfhydryl Analysis in Laboratory Animal Rations

Feeds were extracted with isopropanol fortified with trichloroacetic, ethylenediamine tetraacetic, and diethylenetetramine pentaacetic acids. Pigments in the initial extract and turbidity on aqueous dilution necessitated a cleanup using isooctane after aqueous dilution of the extract. Further aqueous dilution of the isopropanol-water extract insolubilized trace isooctane and more polar lipids. Finally centrifugation in open top tubes removed the last traces of isooctane. In the design of the colorimetry from manual colorimetry (73) the sequence of adding Ellman's reagent before the alkali was reversed to maintain solubility of Ellman's within the autoanalyzer lines. In addition, methanol and aqueous buffer were added to Ellman's reagent to enhance both stability and solubility. Finally the pH of Ellman's reaction was maintained at pH 8.5 rather than at its optimum pH 7 (Section 6.21) to dissolve trace protein haze. Due to the limited selectivity of the colorimetry it was necessary to analyze for and subtract the response of drug- unfortified (blank) feed. The cleanup served to minimize this blank such that captopril could be analyzed down to 0.03% in a variety of animal feeds with good accuracy.

7.72 Semiautomated Ellman Colorimetry for Urinary Captopril and Its Disulfides

The necessity for long term storage of samples prior to analysis and the presence of an oxidation-prone thiol of captopril required development of an acid-chelate stabilization method for urinary captopril (Section 5.22). Thin-layer radiochromatography (Section 7.1) indicated that human urinary captopril was primarily free (unchanged) and, in almost equal proportion,

disulfide-conjugated with cysteine (74). Relatively small amounts of captopril disulfide were observed. An electrochemical reduction (52) was utilized to release disulfide-conjugated captopril for thiol colorimetry. Of several rugged reduction cells evaluated, one with a Vycor® Disc separating the anode and the mercury pool cathode was preferred. Methylene chloride partitions from acidified salt-saturated urines, before and after reduction, allowed the measurement of free and disulfide-conjugated captopril. The drug partitioned into the solvent whereas the aqueous phase retained acid protonated, amino group-bearing thiols like cysteine. Subsequent solvent evaporation volatilized other potential colorimetric interferences. An automated colorimetry of 25 samples per hour was developed by utilizing relatively thiol selective Ellman's Reagent, 5,5'-Dithio-Bis-(2-Nitrobenzoic Acid) (Sections 6.21 and 7.71). Results were confirmed by an HPLC method with electrochemical detection, developed while this method was in use (42).

7.8 High Performance Liquid Chromatography with Electrochemical Detection (HPLC-ECD)

Captopril in urine has been determined by an HPLC-ECD procedure in which the drug is separated from interferences on a monolayer bonded, heavily loaded (about 20% carbon) octadecylsilane reverse-phase column with a mobile phase of 55% methanol and 45% aqueous phosphate buffer (55). A commercially available thin layer mercury film detector was used (Section 6.32). The anodic current, resulting from the oxidation of Hg to Hg-captopril complex, was a linear function of captopril concentration. The peak height was reproducible with a relative standard deviation of 0.54% (n=5). Captopril concentration as low as 0.5 mcg per ml of urine can be quantitated. Sample treatment involved simply centrifugation, filtration, and deoxygenation with nitrogen. Recovery of captopril from urine spiked at 2.0 mcg per ml was better than 95%.

TECHNICON AUTOANALYZER FOR ELLMAN COLORIMETRY

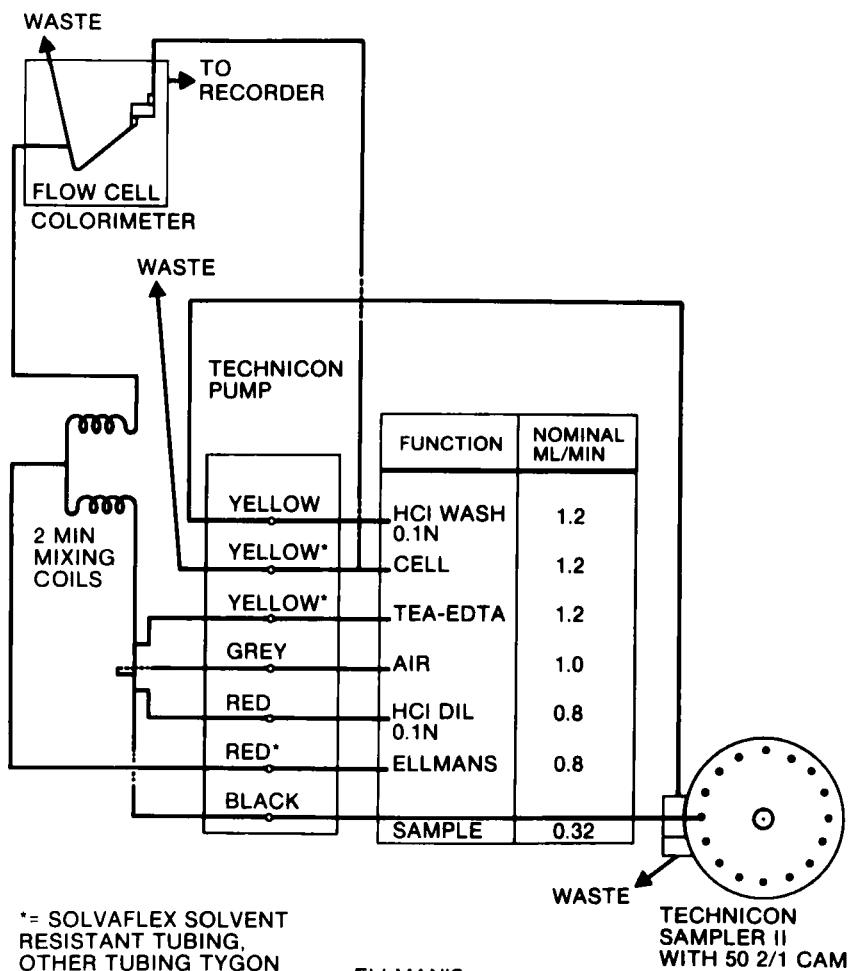


Figure 16

7.9 High-Performance Liquid Chromatography with Fluorescence Detection (HPLC-FD)

Jarrott et al (75) describe an assay for quantitating plasma captopril levels. Blood from patients taking this drug was collected into tubes containing edetate and ascorbic acid, and the plasma was separated by centrifugation. After addition of an internal standard, the plasma was deproteinized and the supernate was adjusted to pH 6.5. N-(1-Pyrene)-maleimide was added to derivatize captopril and an internal standard to fluorescent adducts. These derivatives then were extracted into ethyl acetate-benzene (1:1) and separated from other derivatized thiols by high-performance liquid chromatography. The sensitivity of the assay was 150 pmoles/ml. The HPLC-FD uses a mobile phase of methanol-potassium phosphate buffer (5 mM, pH 6.5) (52:48) and the flow rate was 2 ml/min through a radially compressed octadecylsilane 10 cm x 8 mm (i.d.) column and a spectrofluorometer with a 20 μ l flow cell. Excitation was at 340 nm with emission taken at 390 nm.

8. Drug Metabolism - Pharmacokinetics

Two features salient to the metabolism and pharmacokinetics of captopril include a) the reversible transformation of its thiol function to dimer and mixed disulfide (74,76-79) and b) the biological stability of its amide linkage (80, Section 3). The following will emphasize these aspects.

8.1 Blood Level Studies

Captopril is absorbed rapidly as indicated by measurable blood levels of the drug 15 min after ingestion (81). These findings are compatible with reports of the onset of antihypertensive activity as soon as 15 min after a single oral dose (3) in hypertensive patients and with the rapid onset of blockade of angiotensin I-induced increases in blood pressure in healthy subjects (91). Other studies revealed that the captopril metabolites included its dimer disulfide and the mixed disulfides with glutathione, cysteine, and serum albumin (78,81).

After single oral dosage with 100 mg radioactive captopril, ten healthy subjects attained a mean maximal concentration (C_{\max}) in blood of 800 ± 76 ng/ml at a mean time (T_{\max}) of 0.93 ± 0.08 hours (81,82). Methanol extraction, ultrafiltration, and dithiothreitol reduction studies (77) coupled with TLRC (Section 7.1) established that about 50% of the total blood radioactivity was unchanged captopril, about 10% dimer disulfide and the remainder other polar metabolites including protein and non-protein mixed disulfides (81,82). Thereafter the captopril fraction of total radioactivity declined rapidly with time whereas the mixed disulfide fraction increased. The curvilinearity of semilogarithmic plots of these captopril blood levels with time indicated that half-life ($t_{1/2}$), values could not be accurately calculated. For unchanged captopril levels, curvilinearity may be due to the mixed disulfide fraction acting reversibly to replenish captopril, possibly extending the duration of pharmacologic activity (34,76-79). However plots of total radioactivity allowed calculation of $t_{1/2}$ of 4.3 ± 0.2 hours for the 4-12 hour time interval and 16.4 ± 2.6 hours for the 12-48 hour time interval (81). Mean concentrations of total radioactivity in blood (expressed as captopril equivalents) were 41 ± 7 ng/ml at 24 hours and 20 ± 5 ng/ml at 48 hours after drug administration.

Blood level and urinary excretion studies in 6 hypertensive patients, dosed over a 10 day period, indicated that the metabolic disposition of ^{14}C -captopril was the same at the beginning and end of the study, and was comparable to the disposition in healthy subjects (83). Radiolabeled captopril was administered on days 1 and 10, and blood and urine samples were analyzed for captopril and its metabolites by radiometric assay procedures. Non-radioactive drug (100 mg t.i.d.) was used for dosage on Days 3 to 9.

8.2 Urinary Excretion Studies

In the fasting state, absorption averaged 70-75% of an oral dose, as evidenced by human urinary excretion studies (81,82,84). The presence of food in the gastrointestinal tract

reduces absorption of an oral dose by about 35 to 40% (84).

At least 95% of the radioactivity recovered in urine after a 100 mg oral dose of radiolabelled captopril to mildly hypertensive patients was accounted for as captopril and specific urinary metabolites. Captopril and captopril-cysteine mixed disulfide each accounted for about 45% of the urinary radioactivity (about 33% of the dose) and the disulfide dimer accounted for about 5% of the radioactivity in urine (about 4% of the dose) (74). Additional minor urinary metabolites, in rats and dogs, are S-methyl captopril, captopril-S-methyl sulfoxide, N-acetyl cysteine-captopril and glutathione-captopril mixed disulfides (80). These minor metabolites have not, as yet, been reported in man, but appear to be present in monkeys (90).

Renal dysfunction, as measured by creatinine clearance, decreased the excretion rate of captopril after a single 100 mg oral dose (85). The authors suggest a method of dosage reduction, based on creatinine clearance measurements, in cases of moderate to severe renal dysfunction.

8.3 Miscellaneous Distribution Studies

Biological stability of the amide function was confirmed by studies (80) on rat dermal collagen; uptake of radioactive proline was considerable, whereas insignificant uptake of captopril labelled in the proline moiety was observed.

Whole body radioautography of rats indicated that captopril (50 mg/kg-intravenously) did not readily enter the central nervous system whereas it readily entered the placenta of pregnant rats (86).

Captopril did not readily enter the breast milk of lactating women dosed with captopril at 100 mg t.i.d. for 7 days (87,88). Relatively insignificant milk peak levels were found (4.7 ng/ml).

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I would like to express my appreciation to individuals who have been very helpful for the contributions indicated: Drs. J. Fried and M. Porubcan - NMR, Drs. Y.J. Wang and T. Prusik - Stability, Mr. A. Restivo and Mr. D. Domina - Synthesis and Solubility, Drs. A. Cohen and P. Funke - MS and GC-MS, Ms. M. Malley and Dr. J. Gougatas - Single Crystal X-Ray, Mr. F. Dondzila, Mr. S. Perlman and Dr. J. Kirschbaum - HPLC, Mr. H. Roberts - TLC, Mr. R. Poet and Dr. G. Brewer - Proof Reading and Manuscript Clarity, Ms. D. Walker - Word Processing, Dr. D. Cushman - History, Drs. B. Migdalof and D. McKinstry - Drug Metabolism - Pharmacokinetics.

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11. Review Coverage Dates

This review summarizes communications up to June 10, 1981. However Sections 2 and 8 are updated to about January, 1982.

CEFOTAXIME

Farid J. Muhtadi and Mahmoud M. A. Hassan

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1. Description1.1. Nomenclature1.1.1 Chemical Names

- (a) Sodium 7-[2-(2-amino-4-thiazolyl)-2-methoxyiminoacetamido] cephalosporanate.
- (b) Sodium 3-acetoxymethyl-7-[2-(2-amino-4-thiazolyl)-2-methoxyimino] acetamido]-3-cephem-4-carboxylate.
- (c) (6 R-trans)-3-[(Acetyloxy) methyl]-7-[[(2-amino-4-thiazolyl) (methoxy- imino) acetyl] amino]-8-oxo-5-thia-1-azabicyclo [4.2.0] oct-2-ene-2-carboxylic acid monosodium salt.
- (d) (6-R, 7R)-7-[2-(2-Amino-4-thiazolyl) glyoxylamido]-3-(hydroxy methyl)-8-oxo-5-thia-1-azabicyclo [4,2,0] oct-2-ene-2-carboxylic acid α -(0-methyloxime), acetate (ester) monosodium salt.
- (e) 5-Thia-1-azabicyclo [4.2.0] oct-2-ene 2- \approx carboxylic acid, 3-[(acetyloxy) methyl] -7- \approx [[(2-amino-4-thiazolyl) (methoxyimino) \approx acetyl amino] -8-oxo-, [6R-[6 α ,7 β (Z)]]-

1.1.2 Generic Name

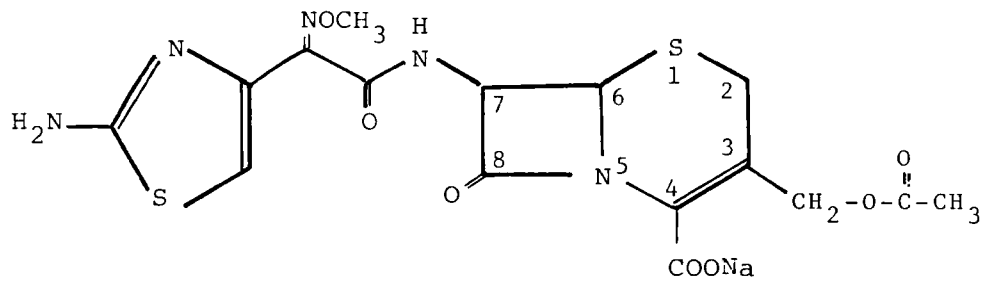
Cefotaxime sodium; HR 756; RU-24,662;
RU-24,756

1.1.3 Proprietary Names

Claforan; Primafen; Zariviz; Tarivid.

1.2. Formula1.2.1 Empirical $C_{16}H_{16}N_5O_7S_2Na$

1.2.2 Structural



1.2.3 CAS no.

[64846-23-3] (1)

 $C_{16}H_{16}N_5O_7S_2Na$

[60846-21-1] (1)

 $C_{16}H_{17}N_5O_7S_2$ (as free acid)

[63527-52-6] (2)

1.3. Molecular Weight

477.23

1.4. Elemental Composition

C, 40.23%; H, 3.38%; N, 14.67%;

O, 23.47%; S, 13.44%; Na 4.82%.

1.5. Appearance, Color, Odor and Taste:

White to creamy white crystalline powder,
odorless and has a salty taste at the beginning,
followed by bitterness.

2. Physical Properties2.1. Solubility

Freely soluble in water (0.5g soluble in 5 ml) (3),
slightly soluble in alcohol (absolute, 95%),
insoluble in chloroform (4)

2.2. Moisture Content

Not more than 6 percent, determined by the Karl
Fischer method, using a 0.2 g sample dissolved
in 2 ml methanol (3).

Loss on drying at 60°C under vacuum in the
presence of phosphorus pentoxide for 4 hr.
should not exceed 6 % (3).

2.3. pH range

The pH of cefotaxime as a 10% aqueous solution
is 4.5 to 6.5 determined potentiometrically (3).

2.4. Optical Rotation

$[\alpha]_D$ (c=1% aqueous solution) + 58 to + 64°
(on dried bases) (3).

The optical rotation of cefotaxime (c=1%
aqueous solution) was determined using a
Perkin Elmer 241 MC Polaromatic and found to be:

$$[\alpha]_D^{24^\circ} + 59.3^\circ.$$

2.5. Spectral Properties

2.5.1 Ultraviolet Spectra

The UV spectra of cefotaxime in an aqueous solution and in 0.1N HCl are presented in Fig. 1 and Fig. 2 respectively. These were scanned from 200 to 400 nm using a Pye-Unicum SP 8-100 Ultraviolet spectrophotometer. The following table 1 shows the UV data.

Table 1. UV characteristics of cefotaxime

<u>solvent</u>	<u>concentration</u>	<u>max.nm</u>	<u>E(1%,1cm)</u>
water	0.5 mg/ml	234	
0.1N HCl	0.05 mg/ml	205,262	
0.1N HCl	0.01 mg/ml	263	420 (5)

2.5.2 Infrared Spectra

The IR spectra of cefotaxime as KBr-disc and as nujol mull are shown in Fig. 3 and Fig. 4 respectively. The KBr-disc was recorded on a Perkin Elmer 580B infrared spectrometer. The structural assignments have been correlated with the following band frequencies (Table 2).

Table 2. IR characteristics of cefotaxime

<u>Frequency Cm^{-1}</u>	<u>Assignment</u>
3420	$-\text{NH}_2$
3340 (broad)	$-\text{NH}$, $-\text{NH}_2$
2940	$-\text{S}-\text{CH}_2$
1760	$-\text{C}=\text{O}$ lactam $\overset{\text{O}}{\parallel}$
1730	$-\text{C}=\text{O}$ carboxylic, $\overset{\text{O}}{\parallel} \text{O}-\text{C}-\text{CH}_3$
1650	$-\overset{\text{O}}{\parallel}\text{C}-\text{NH}$
1620	$-\overset{\text{O}}{\parallel}\text{C}-\text{NH}$, $-\text{C}=\text{N}-$, $-\text{C}=\text{C}-$
1540	$-\overset{\text{O}}{\parallel}\text{C}-\text{N}-$
1385-1355	$-\text{O}-\text{CO}-\text{CH}_3$
1180	$\text{C}=\text{O}$ in ester
1050	$\text{C}-\text{O}$ stretching

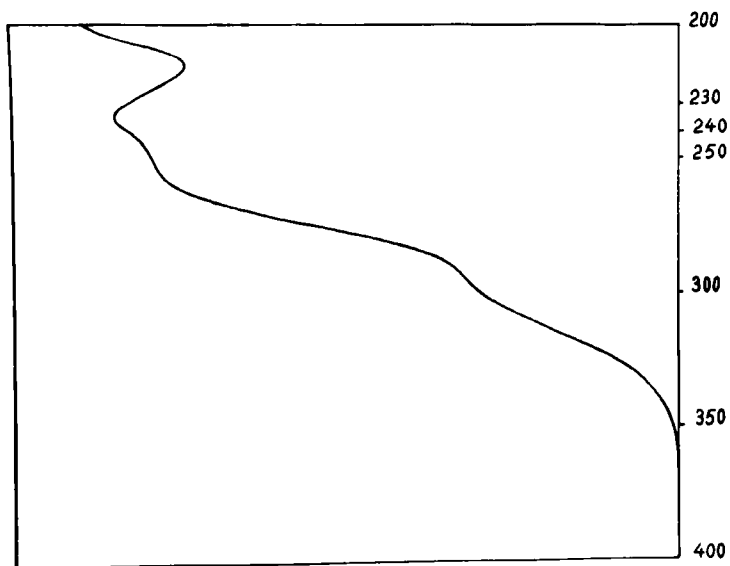


Fig.1 The UV spectrum of Cefotaxime in water .

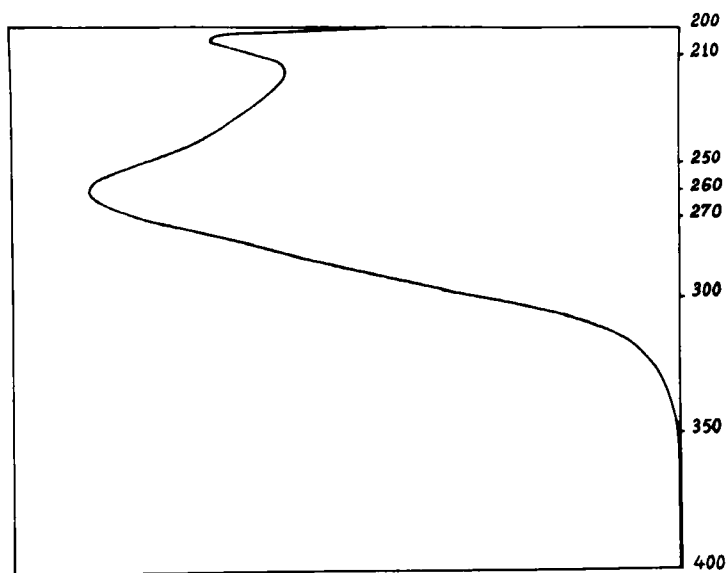


Fig.2 The UV spectrum of Cefotaxime in 0.1N HCl .

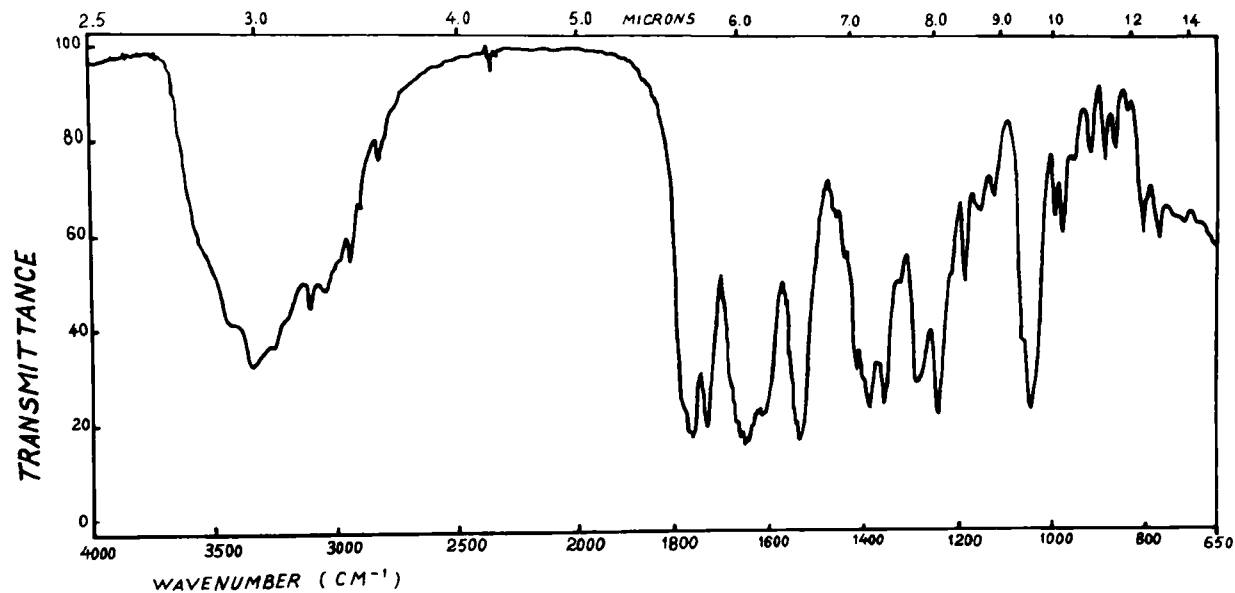


Fig. 3. The IR spectrum of Cefotaxime in KBr disc.

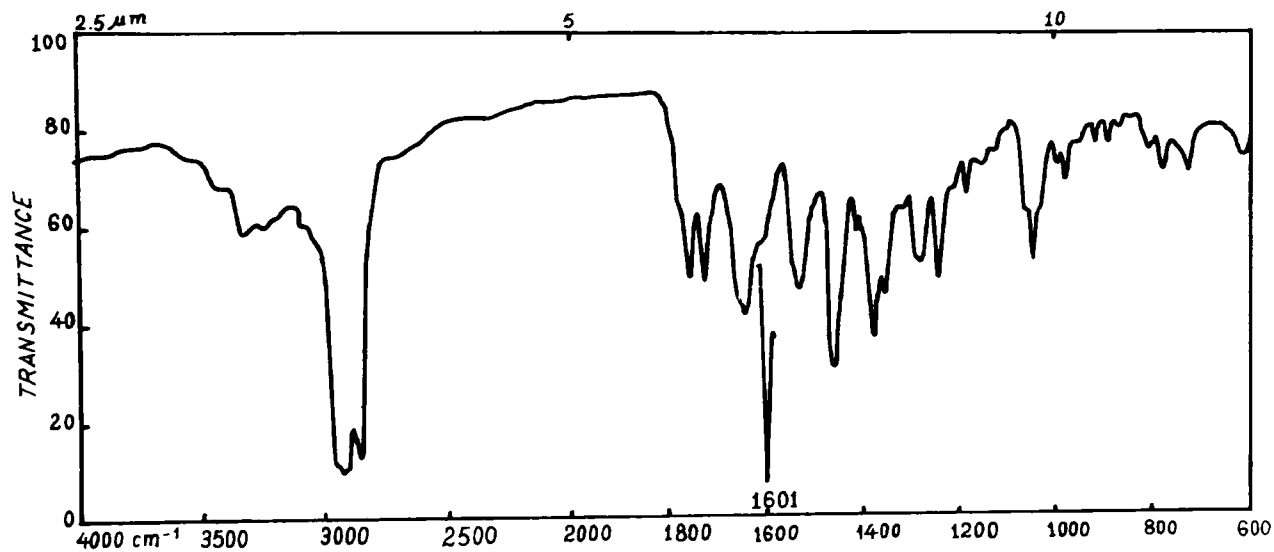


Fig. 4. The IR spectrum of Cefotaxime in nujol mull.

2.5.3 Nuclear Magnetic Resonance Spectra

2.5.3.1 Proton Spectra

The PMR spectra of cefotaxime in deuterated dimethyl sulfoxide (DMSO-D₆) and deuterium oxide were recorded on a varian T-60A, 60-MHz NMR spectrometer using sodium -2, 2-dimethyl-2-silapentane-5-sulfonate (DSS) as an internal reference. These are shown in Fig. 5 and Fig. 6 respectively.

The following structure assignments have been made (Table 3) (6).

Table 3. PMR characteristics of cefotaxime

<u>Group</u>	<u>Chemical Shift (ppm)</u>	
	DMSO-D ₆	D ₂ O
-OCO <u>CH</u> ₃	2.00(s)	2.12(s)
2- <u>CH</u> ₂	3.33(q)	3.53(q)
N- <u>OCH</u> ₃	3.83(s)	4.00(s)
O- <u>CH</u> ₃ Ac.	4.97(q)	4.83(d)
6-H	4.97(q)	5.20(d)
7-H	5.60(2d)	5.82(d)
5-H	6.70(s)	6.97(s)
2'- <u>NH</u> ₂	7.22(bs)	-
<u>CONH</u>	9.47(d)	-

s=singlet, d=doublet, q=quartet, 2d=doublet of doublets, bs=broad singlet.

2.5.3.2 ¹³C-NMR

¹³C-NMR completely decoupled and off resonance spectra are presented in Fig. 7 and Fig. 8 respectively. Both were recorded over 5000 Hz range in deuterium oxide (conc. 100 mg/1 ml D₂O)

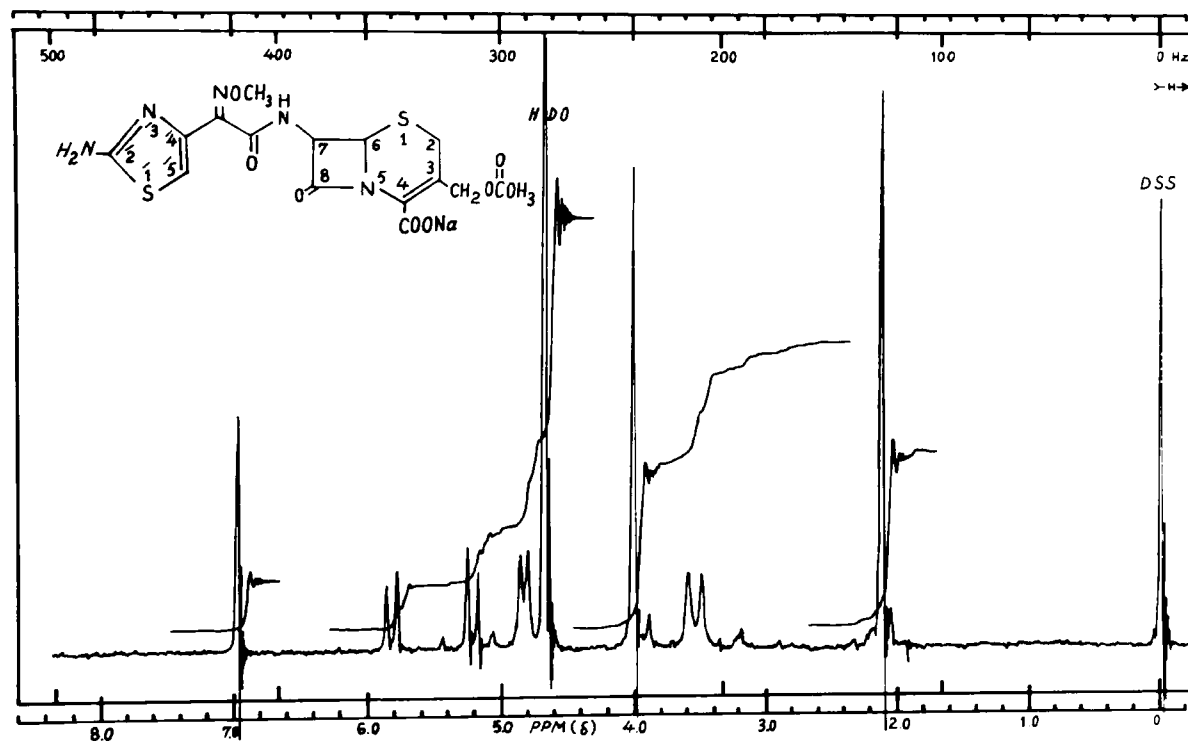


Fig. 5. The PMR spectrum of Cefotaxime in D_2O

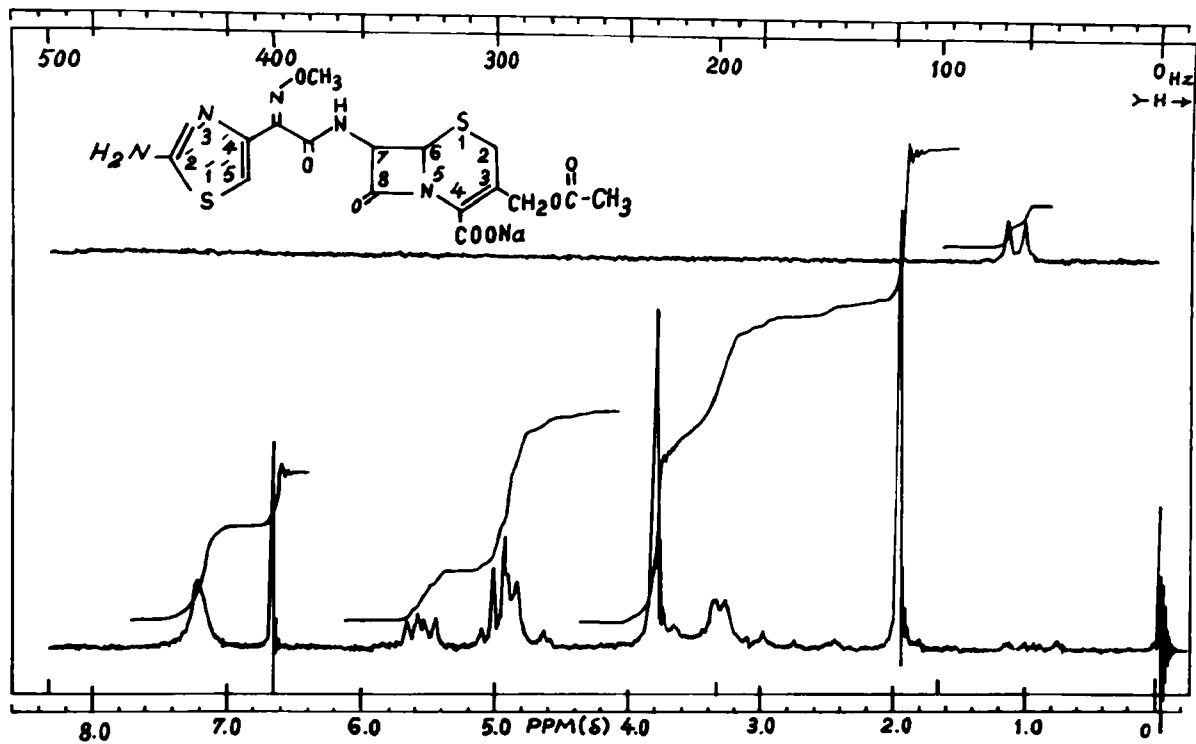


Fig. 6. The PMR spectrum of Cefotaxime in DMSO D_6

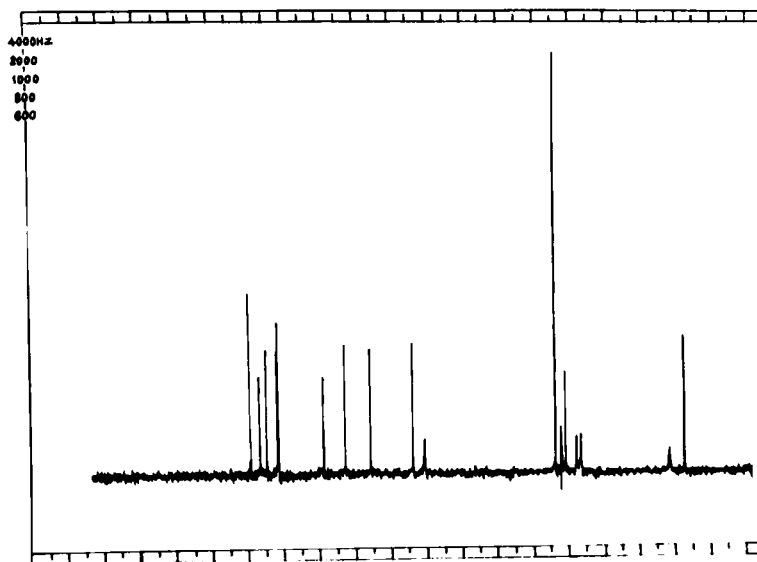


Fig. 7. The ^{13}C NMR of Cefotaxime, completely decoupled spectrum.

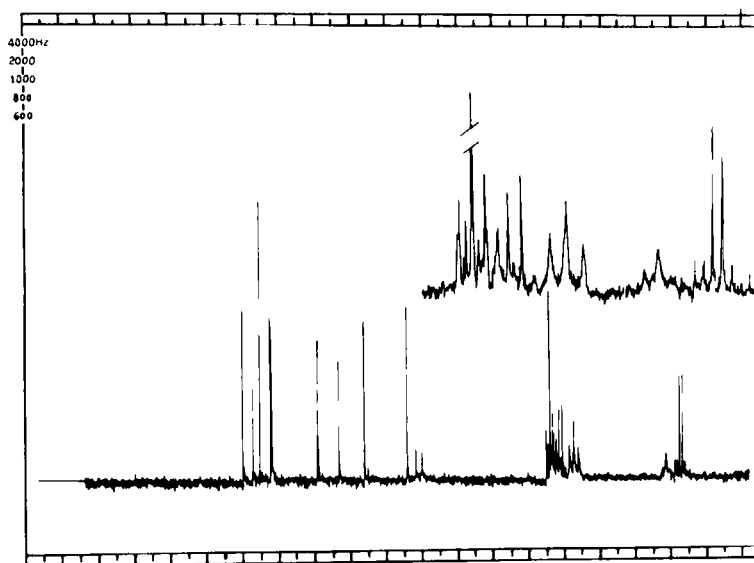


Fig. 8. The ^{13}C NMR of Cefotaxime, off resonance (Proton coupled) spectrum

on FT-80A-80MHz NMR spectrometer, using 10 mm sample tube and dioxane as a reference standard at 20°C. The carbon chemical shift are assigned on the basis of the additivity principals and the off resonance splitting pattern (Table 4) (6).

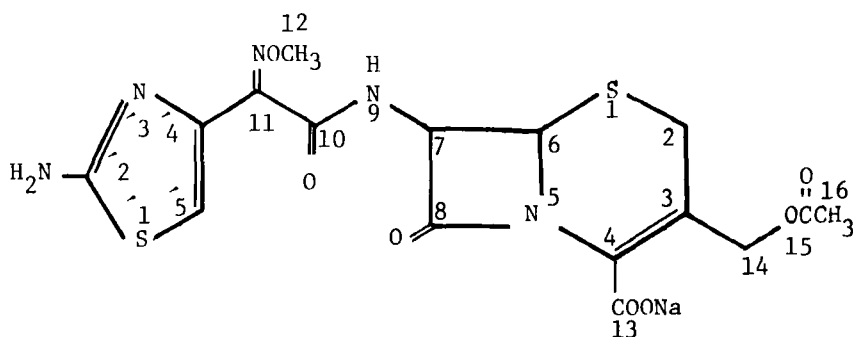


Table 4. Carbon Chemical shifts of cefotaxime

Carbon no.	Chemical shift [ppm]	Carbon no.	Chemical shift [ppm]
8-C=O	174.69(s)	C-3	117.37(s)
10-C=O	171.25(s)	C-5	113.64(d)
13-C=O	168.78(s)	C-7	65.07(d)
15-C=O	165.08(s)	C-6	59.59(d)
C-2	164.65(s)	C-14	58.23(t)
C-11	148.62(s)	C-2	26.35(t)
C-4	141.40(s)	C-12	63.55(q)
C-4	132.42(s)	C-16	21.22(q)

s=singlet, d=doublet, t=triplet, q=quartet.

3. Synthesis of Cefotaxime

Cefotaxime is a semi-synthetic cephalosporin. The starting material for such cephalosporins is 7-aminocephalosporanic acid (7-ACA) which obtained either by mild acid or enzymatic hydrolysis of cephalosporin C. (7,8,9,10)

Preparation of the Side Chain: (5)

The starting material ethylacetoacetate [1] was oxidized to produce oximinoethylacetoacetate [2]. Methylation of [2] followed by bromination yielded syn-methoxyiminobromoketone [3]. Condensation of [3] with thiourea [4] in aqueous medium and at a low temperature gave syn-aminothiazolyl derivative [5]. The aminothiazolyl ring was protected by tritylation to give the N-trityl derivative [6]. Saponification of the latter by boiling with NaOH solution afforded the corresponding acid [7]. Acylation of the amino group of 7-ACA with the resulting acid [7] proved difficult. This has been overcome by the use of symmetrical anhydride [8], which was obtained by condensing two molecules of [7] in the presence of dicyclohexylcarbodiimide [a]. 7-ACA was acylated by compound [8] to give [9]. Removal of the trityl residue under acidic condition gave the free acid form (R=H) of cefotaxime [10].

Preparation of a Stable Sodium Salt:

This was prepared by adding the solid free acid to an aqueous alcoholic solution of an organic sodium salt to give the stable active D-form. (5)

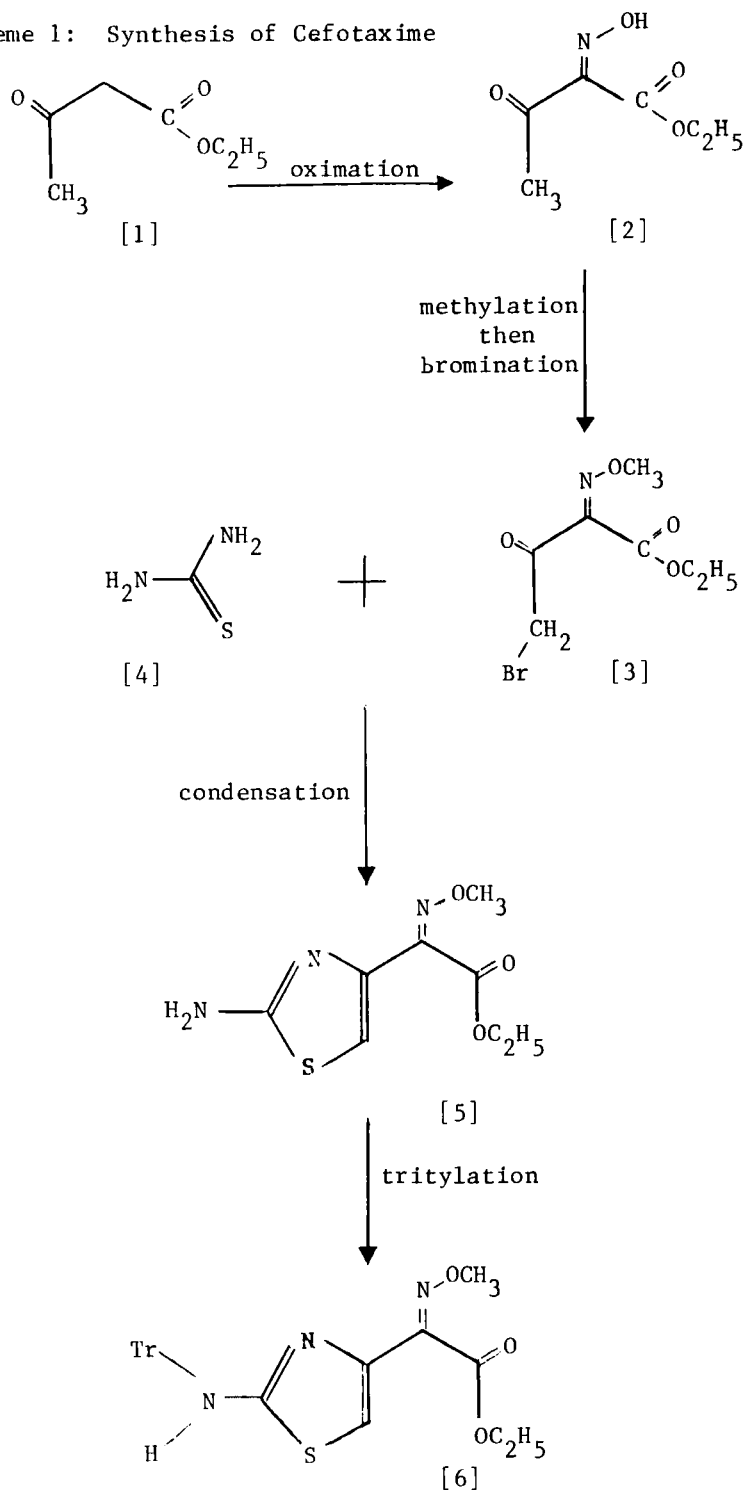
The synthesis of cefotaxime is presented in Scheme 1.

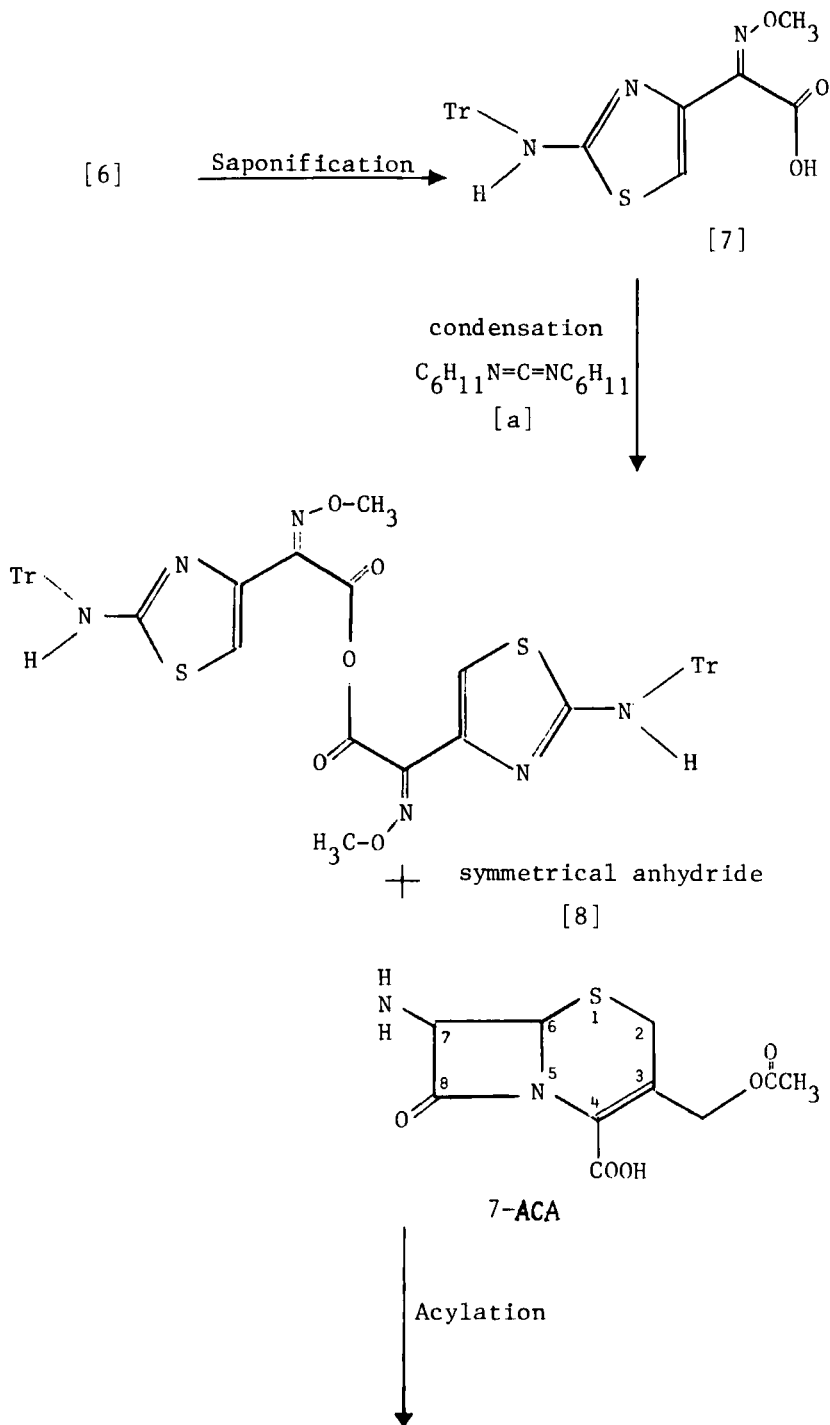
It is interesting to note that the syn-isomer of cefotaxime is up to 100 times more active against certain organisms than the anti-isomer.

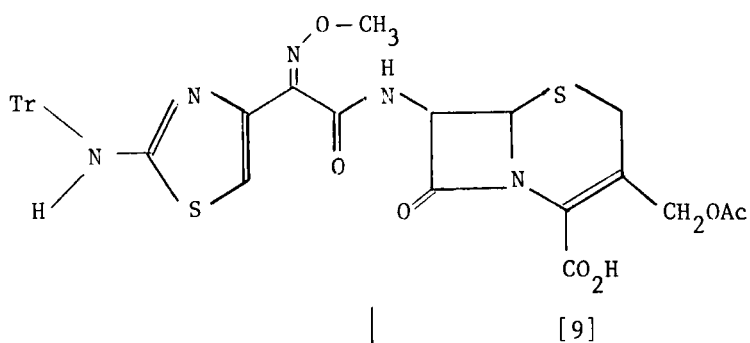
4. Metabolism

The metabolism of cefotaxime in rat, dog and man using ^{14}C -labelled cefotaxime has been studied by Chamberlain et al. (11). Cefotaxime is well absorbed in the three species after intramuscular administration. It is eliminated mainly via the urine. The major metabolite being desacetyl cefotaxime. The amount of unchanged cefo-

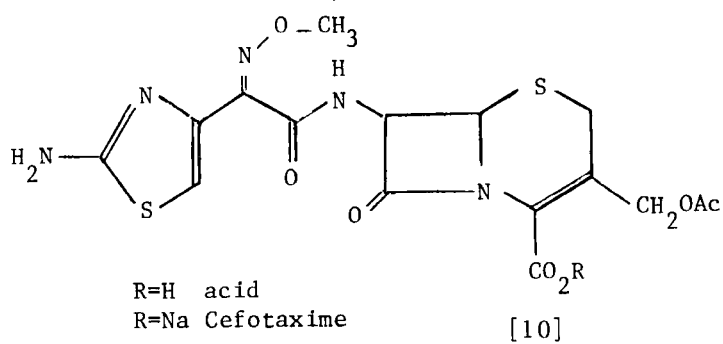
Scheme 1: Synthesis of Cefotaxime







acidification



taxime and the major metabolite eliminated in the urine is similar for each species. Two further metabolites, UP1 and UP2 are detected in dog and human urine but not in rat urine.

The formation of both UP metabolites is related to the rate of renal elimination of desacetyl cefotaxime (11).

The metabolic pathway follows the route in dog and human which may occur in the liver is presented in Scheme 2.

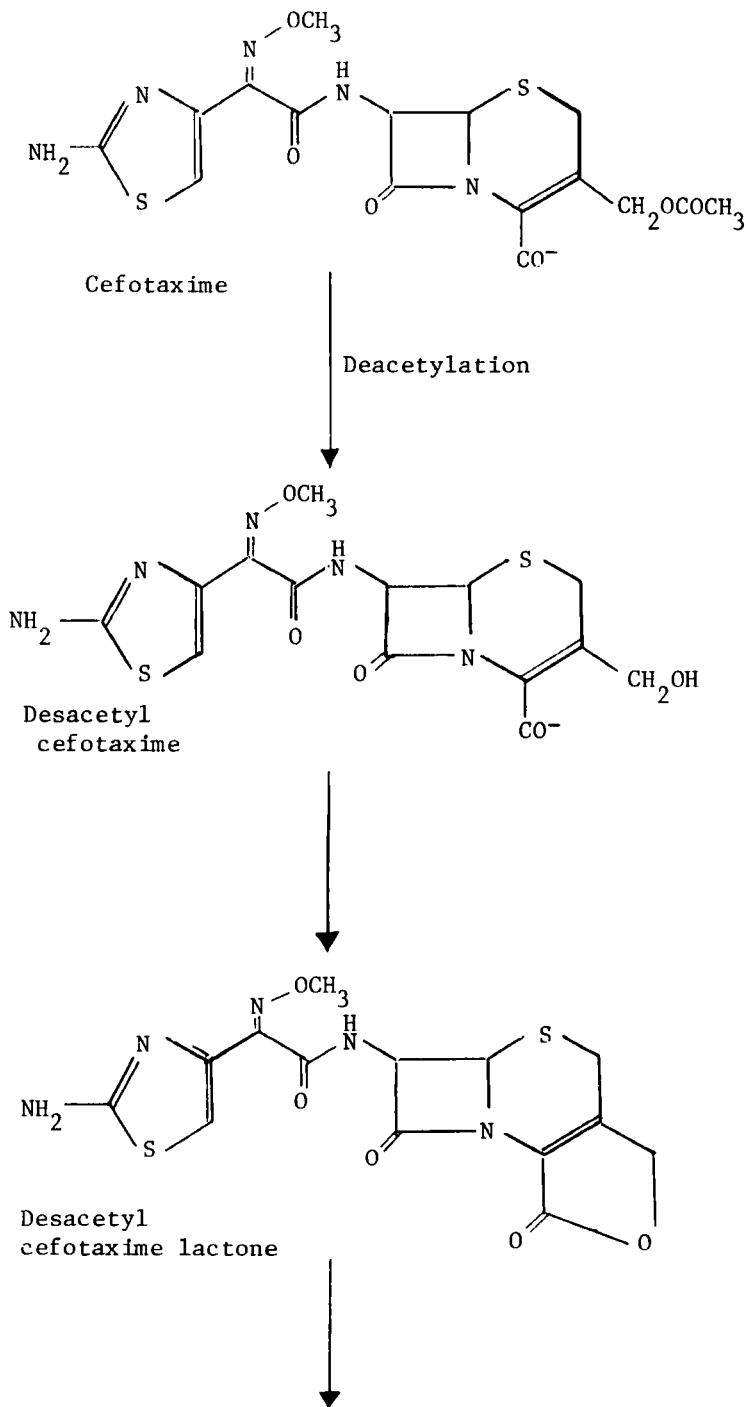
5. Pharmacokinetics

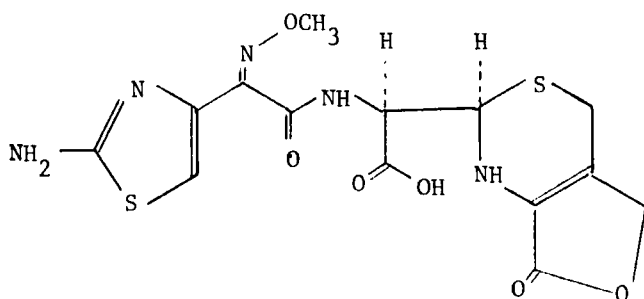
The pharmacokinetics of cefotaxime was reported to be dose independent for doses up to 2.0 g (12). Following the intramuscular (i.m.) injection of a 0.5 g (13) and 1.0 g doses (12), the mean peak serum level of 11.7 $\mu\text{g/ml}$ and 22 $\mu\text{g/ml}$ at 0.5 h were obtained respectively. After intravenous (i.v.) bolus injection of 1.0 g cefotaxime, the mean peak level was 105 $\mu\text{g/ml}$ and an apparent volume of distribution of 25 litres was estimated (12). Values of 32 and 37 litres for the volume of distribution were also reported (13). Cefotaxime is mainly eliminated by renal excretion and serum-half-life ranged from 0.92 to 1.35 h after 1 g i.m. injection, and from 0.84 to 1.25 h after i.v. administration (12). The majority of the dose being excreted unchanged in the urine ($\approx 51\%$ within 8 h) (12). In another study (13) the serum clearance of 341 ml/min. per 1.73 m^2 and renal clearance of 130 ml/min per 1.73 m^2 were obtained. Protein binding of cefotaxime ranged from 35 to 45%.

6. Microbiological Activity

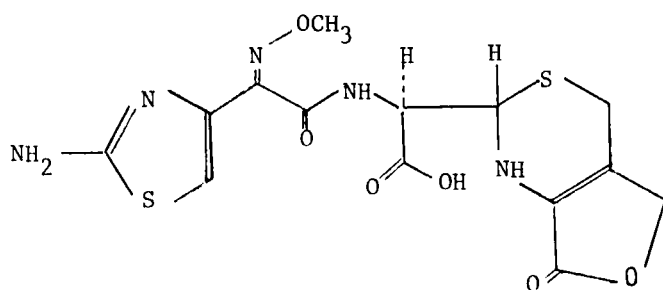
Cefotaxime possesses an extremely broad antibacterial spectrum and is very active against G positive bacteria especially on Enterobacteriaceae, Haemophilus influenzae or Neisseria gonorrhoeae. It is also active on Bacteroides fragilis and Pseudomonas aeruginosa (14). Cefotaxime penetrates well through the cell walls, showing high β -lactamase stability and strong affinity to the important target enzymes (14).

Scheme 2: Metabolism of Cefotaxime.





UP1



UP2

7. Methods of Analysis

7.1. Identification Tests

The following identification tests are mentioned under cephalixin in the B.P. 1980 (15) :-

- A) The infra-red absorption spectrum determined in the solid state, is concordant with the reference spectrum of cefotaxime.
- B) Mix 20 mg with a few drops of an 80 % V/V solution of sulfuric acid containing 1 % V/V of nitric acid; a yellow color is produced.
- C) Mix 20 mg with 5 drops of a 1 % V/V solution of glacial acetic acid and add 2 drops of a 1 % W/V solution of copper sulfate and drop of 2M sodium hydroxide; an olive-green color is produced.

7.2. Non-Aqueous Titration Assay

Weigh accurately about 0.15 g of cefotaxime and dissolve in 50 ml glacial acetic acid. Assay potentiometrically with 0.1 N perchloric acid.

1 ml of 0.1 N perchloric acid corresponds to 23.87 mg of cefotaxime (3).

7.3. Chromatography

7.3.1 Thin Layer Chromatography (TLC)

TLC of cefotaxime is performed on silica gel coated, self-indicating chromatographic plates (60 F 254, Merck).

The developing solvent system is:-

Ethylacetate/Ethanol/Water/Formic acid
(60 : 25 : 15 : 1) by volume.

Development is carried out in a lined tank, and the solvent is allowed to migrate about 15 cm from the starting points.

The plates are visualized by examining under ultraviolet light at 254 nm (3).

The above method can be adopted for the determination of total impurity content of cefotaxime as follows (3) :-

A 2 % solution of the sample to be determined is dissolved in a mixture of 8 vol. acetone and 2 vol. water. Cefotaxime reference substance (HR 756 standard) is dissolved in the same mixture to produce 0.02 %, 0.04 % and 0.08 % solutions.

Two spots 0.01 and 0.02 ml of the tested solutions are applied to the chromatoplate these representing 200 and 400 µg respectively.

A 0.01 ml of each standard solution is also applied to the chromatoplate representing 2, 4, 8 µg respectively. The plate is developed as above. The total impurity content should not exceed 5 %.

Another TLC system is recommended for cefotaxime as follows:-

A solution of cefotaxime is applied into a silica gel G chromatoplate. The plate is developed in the solvent Methanol : Ammonia (100 : 1.5). After development, the plate is air dried, sprayed with 0.5 % iodine in chloroform. Cefotaxime developed a dark brown spot which has R_f value of 0.83 (4)

7.3.2 Paper Chromatography

Descending paper chromatography was accomplished using Whatman no. 1 paper. The solvent system consisted of isopropanol-pyridine-water (65 : 5 : 30, V/V). The chromatogram was subjected over-night for development. Examination was conducted under the UV light at 254 nm.

7.3.3 Gas Liquid Chromatography (GLC)

A GLC method for the determination of cefotaxime has been adopted in our laboratory

using a Varian GC - 3700 gas chromatograph equipped with Varian CDS 111 integrator.

Column conditions : 3 % OV-17 on chromosorb WHP (80 - 100 mesh) glass column (2 m x 2 mm)
The column was conditioned isothermally at 120° for 10 min. and then the temperature was programmed at 10°/min up to 210°.

Carrier gas : Helium, flow rate was adjusted to 20 ml/min.

Detector : FID, hydrogen and air flow rates were adjusted to 40 ml/min. and 300 ml/min. respectively.

Ethanol was used as the solvent and the chart speed was adjusted to give 0.5 cm./min.

The retention time = 8.15 min.

The GLC of cefotaxime is presented in Fig. 9.

Another GLC method has been described for the determination of residual solvents in cefotaxime (3).

Apparatus : A 1.5 m PORAPAK Q column programed at 150° with a flame ionization detector was used. Isopropanol was used as the internal standard.

Solution I : 70 mg cefotaxime standard (HR 756) and 0.08 mg isopropanol were dissolved in water to produce 1 ml.

Solution II : 0.05 mg methanol, 0.13 mg ethanol and 0.08 mg isopropanol were mixed with water to produce 1 ml.

Procedure : Solutions I and II were injected and the solvent levels were determined from the peak heights corrected against the internal standard.

Results : Methanol should not exceed 0.2 % ethanol or any other organic solvent should not exceed 1 %.

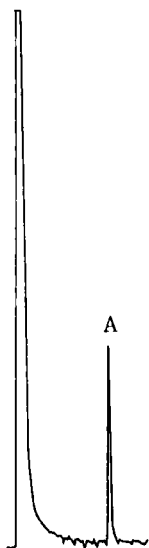


Fig 9. GLC of cefotaxime

A = cefotaxime

7.3.4 High Performance Liquid Chromatography (HPLC)

Several HPLC systems for the identification and separation of cefotaxime and its metabolites have been reported. These systems are given in Table 5.

HPLC of cefotaxime and its metabolites using system 1 (11) is presented in Fig. 10.

Table 5. HPLC Systems of Cefotaxime

System no.	Column	Mobile Phase	Retention Time (min)	Detection (nm)	Ref.
1.	A nucleosil/ ODS column	Acetonitrile- water-acetic acid (8:91:1 by vol)	13.0	UV region	(11)
2.	A Microbondapak, C ₁₈ corasil guard column	water-methanol (39 : 7) containing 0.005 M heptane sulfonic acid	30.0	254 nm	(16)
3.	Si-C ₁₈ , 10 microns	Methanol-water- (1 : 5) KH ₂ PO ₄ 0.06 % Na ₂ HPO ₄ 2H ₂ O to reach pH 7.8	-	235 nm	(3)
4.	Lichrosorb RP-8	Phosphoric acid- methanol	17.0	310 nm	(17)

Table 5 contd.

System no.	Column	Mobile Phase	Retention Time (min)	Detection (nm)	Ref.
5.	A Reversed-phase Hibar RT 250-4 Lichrosorb RP 18-7 μm , corasil C ₁₈ 37-50 μm guard column	20 m mol sodium dihydrogenphosphate in water-methanol- acetonitrile (83:7:10, V/V/V)	4.8	254 nm	(18)
6.	A 10-cm octade- cylsilane column and a 4-cm pre- column containing copellicular octadecylsilane	14% methanol, 1% acetic acid, in distilled water	12.0	254 nm (set at 0.005 absorbance unit)	(19)

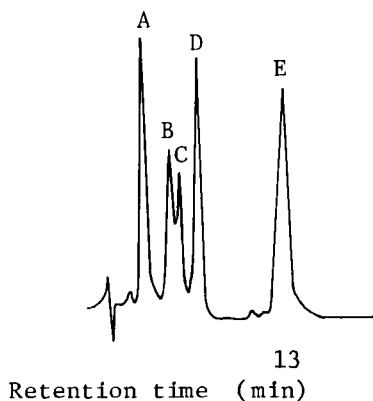


Fig. 10 HPLC of Cefotaxime and its Metabolites
A, Desacetyl cefotaxime; B, UP1; C, UP2;
D, Desacetyl cefotaxime lactone; E, cefotaxime.

7.4 Spectrophotometry

A PMR method for quantitative determination of cefotaxime and other cephalosporins in bulk drugs and various dosage forms is reported (20). The determination is based on the integration of the 6-H and / or 7-H of the β -lactam ring system relative to that of the nine protons of t-butanol. The method is rapid, accurate and precise, with an average standard deviations of ± 1.51 in standard mixtures and ± 1.15 in pharmaceutical formulations. The procedure furnishes a specific means of identification of each individual cephalosporin as well as detection of the commonly encountered impurities.

Procedure:

Weigh accurately a portion of the powder equivalent to 100 mg of the cephalosporin into a glass stoppered sample tube. Add 1.0 ml accurately measured D_2O containing an accurate weight of t-butanol, stopper and shake for 3 min. Transfer about 0.5 ml of the resulting solution into a NMR tube and record the spectrum, adjusting the spin rate to reduce the spinning side bands as

much as possible. Integrate the peaks of interest (The 6- or 7-H of the β -lactam ring appearing at 4.86 - 5.80 ppm and the 9 protons of t-butanol appearing at 1.23 ppm) at least three times and determine the average integrals.

The amount of cephalosporin is then calculated as follows:-

$$\text{mg of cephalosporin} = \frac{A_c}{A_b} \times \frac{E.W_c}{E.W_b} \times \text{mg of t-butanol}$$

Where A_c is the integral value of the cephalosporin signal, A_b that of the t-butanol signal, $E.W_c$ is the molecular weight of cephalosporin and $E.W_b$ is one ninth of the molecular weight of t-butanol (= 8.24).

7.5 Microbiological Assay

Cefotaxime is microbiologically assayed by the agar well diffusion method of Grove and Randall (21) as modified by Chabbert and Boulenger (22).

The following table 6 summarises the media and the test organisms as reported.

Table 6. Media and Test Organisms Used

<u>Medium</u>	<u>Test Organism</u>	<u>Ref.</u>
1. Agar antibiotic no.2 (Difco)	Spores of <u>Bacillus subtilis</u>	(23)
2. " "	<u>Bacillus subtilis</u> (ATCC 6633)	(24)
3. " "	<u>Sarcina leutea</u> (ATCC 9341)	(24)
4. " "	<u>Escherichia coli</u> 3989	(13)
5. " "	<u>E. coli</u> (ATCC 9637)	(26)
6. " "	<u>Proteus morganii</u>	(19)
7. Antibiotic no.3 (Oxoid)	<u>Staphylococcus epidermidis</u> Q 305	(25)
8. Mueller-Hinton agar	<u>Proteus mirabilis</u> (ATCC 14273) <u>Pseudomonas aeruginosa</u> (K 1118)	(18)

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Acknowledgment

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CEFOXITIN, SODIUM

Gerald S. Brenner

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1. Introduction

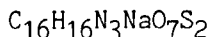
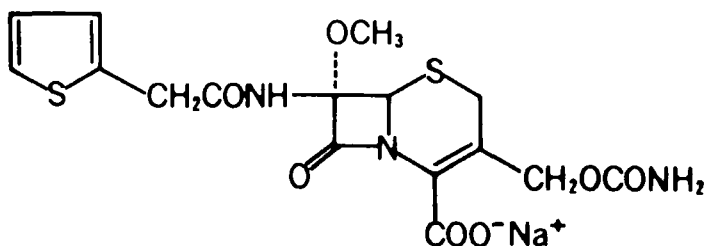
1.1 History

Sodium cefoxitin, a semi-synthetic cephamycin antibiotic was synthesized, tested and developed by the Merck Sharp and Dohme Research Laboratories. Cefoxitin is active against Gram-positive and Gram-negative bacteria (1) and is therapeutically important because of its resistance to destruction by bacterial β -lactamase (2,3,4).

1.2 Name, Formula, Molecular Weight

The Chemical Abstracts name for sodium cefoxitin (MK-306) is 3-[[[Aminocarbonyl]oxy]methyl]-7-methoxy-8-oxo-7[(2-thienylacetyl)amino]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid sodium salt. The CAS registry no. is 33564-30-6.

Other names include 3-carbamoyloxymethyl-7 α -methoxy-7 β -(2-thienylacetamido)-3-cephem-4-carboxylic acid sodium salt, sodium 3-carbamoyloxymethyl-7-methoxy-7-[2-(2-thienyl)acetamido]-3-cephem-4-carboxylate and sodium (6R,7R)-3-(hydroxymethyl)-7-methoxy-8-oxo-7-[2-(2-thienyl)acetamido]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2 carboxylate carbamate (ester).



Molecular Weight: 449.44

1.3 Definition

Sodium cefoxitin, unless specified otherwise, is defined as the crystalline, non-solvated salt form of the compound. Cefoxitin when specified refers to the free acid.

1.4 Appearance, Color, Odor

White to off-white granules or powder having a slight characteristic odor.

2. Physical Properties

2.1 Infrared Spectrum

The infrared spectrum of cefoxitin presented in Figure 1 was taken in a KBr pellet using a Perkin Elmer Model 621 infrared spectrophotometer. A list of the assignments made for some of the characteristic bands is given in Table I (5).

Table I

IR Spectral Assignments for Cefoxitin

<u>Frequency (cm⁻¹)</u>	<u>Assignment</u>
3200-3500	various N-H stretch
3000 (broad)	OH stretch
1780	β -lactam C=O stretch
1715	carboxyl C=O stretch
1680	carbamate C=O stretch
1660	amide C=O stretch
1420	various -CH ₂ bends
1080	carbamate C-O stretch

The infrared spectrum of sodium cefoxitin taken as a mineral oil mull is shown in Figure 2.

2.2 Magnetic Resonance Spectra

2.21 Proton Spectrum

The proton magnetic resonance spectrum of cefoxitin presented in Figure 3 was obtained using a Varian A-60A spectrometer with DMSO-d₆ as the solvent. The spectral assignments are given in Table II (6). The proton magnetic resonance spectrum for sodium cefoxitin in DMSO-d₆ is shown in Figure 4.

Table II

Proton Magnetic Resonance Assignments for Cefoxitin

<u>ppm (δ)</u>	<u>Relative Intensity</u>	<u>Multiplicity</u>	<u>Assignment</u>
9.38	1	Singlet	O -C-NH-
7.34	1	Multiplet	thienyl- α
6.85-7.05	2	Multiplet	thienyl- β

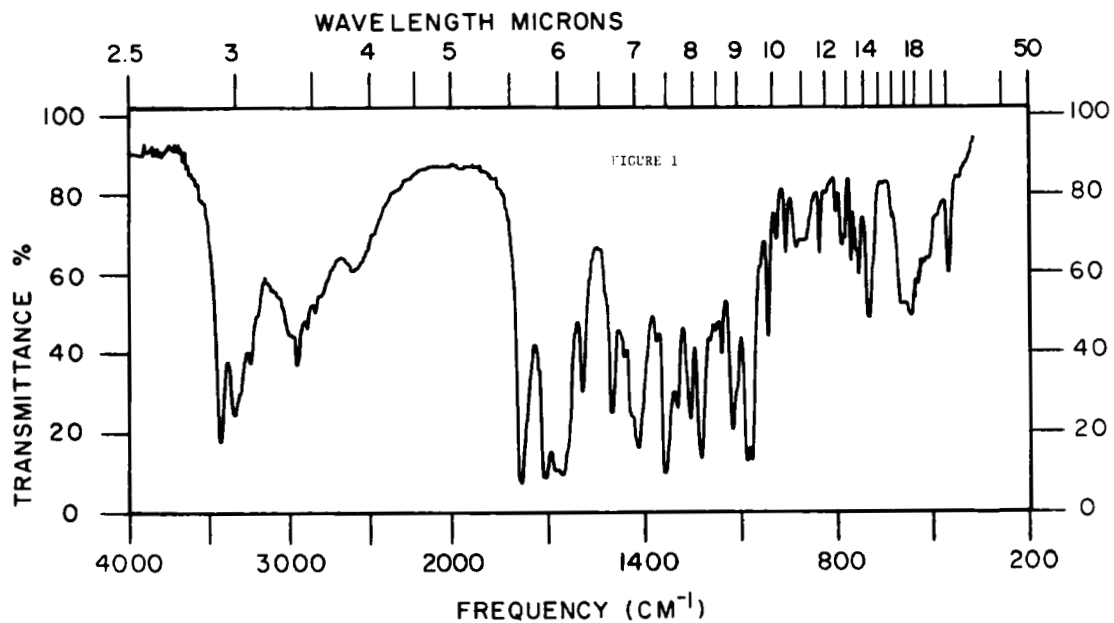


Figure 1. Infrared Spectrum of Cefoxitin, KBr Pellet

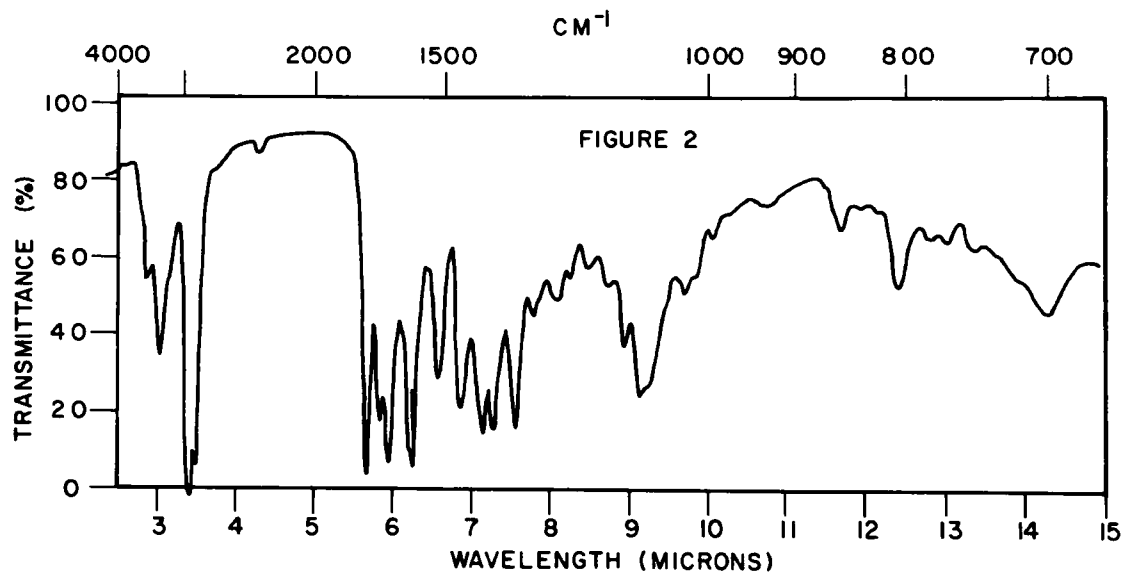


Figure 2. Infrared Spectrum of Sodium Cefoxitin, Mineral Oil Mull

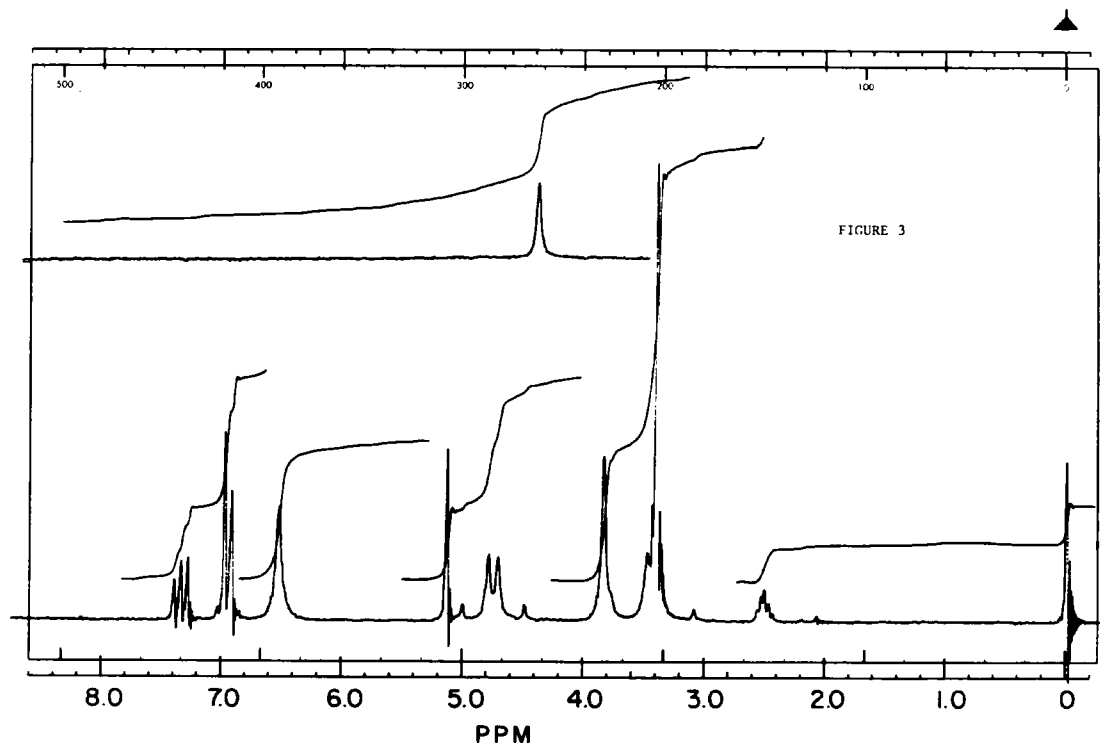


Figure 3. Proton Magnetic Resonance Spectrum of Cefoxitin in DMSO-d₆.

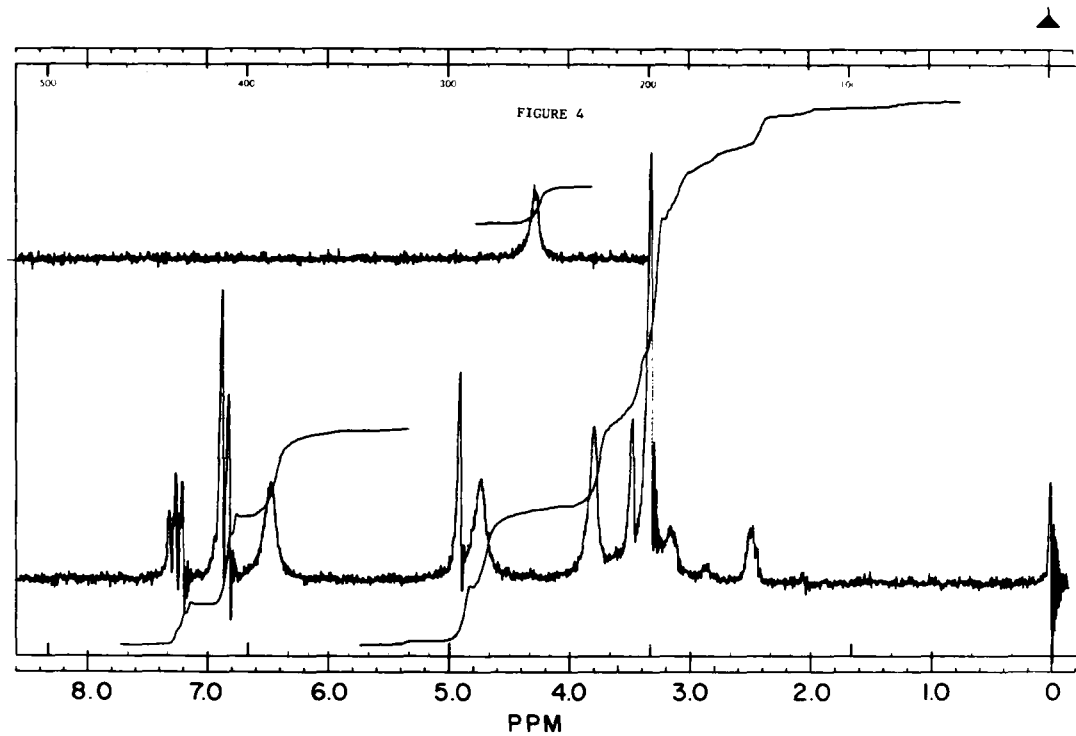


Figure 4. Proton Magnetic Resonance Spectrum of Sodium Cefoxitin in DMSO-d₆.

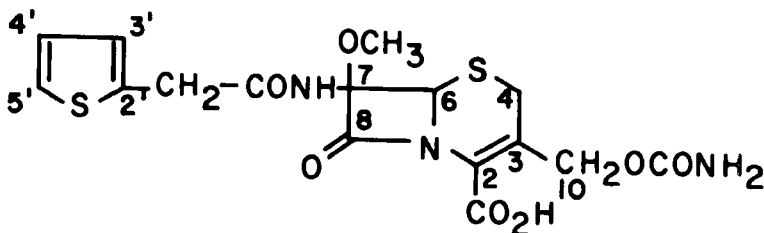
Table II (cont'd)

<u>ppm (δ)</u>	<u>Relative Intensity</u>	<u>Multiplicity</u>	<u>Assignment</u>
6.52	2	Singlet	$\begin{array}{c} \text{O} \\ \\ \text{O}-\text{C}-\text{NH}_2 \end{array}$
5.13	1	Singlet	6-H
4.49-5.0	2	Multiplet	$\begin{array}{c} \text{O} \\ \\ -\text{CH}_2\text{OC}- \end{array}$
$\left. \begin{array}{l} 3.83 \\ 3.08-3.80 \\ 3.40 \end{array} \right\}$	6-7	Singlet Multiplet Singlet	$\left\{ \begin{array}{l} \text{thienyl}-\text{CH}_2-\text{C} \begin{array}{c} \text{O} \\ \end{array} \\ 4-\text{CH}_2 \\ 7-\text{OCH}_3 \end{array} \right.$

2.22 Carbon-13 Spectrum

The carbon-13 magnetic resonance spectra of cefoxitin presented in Figures 5 and 6 were obtained using a Varian CFT-20 spectrometer with DMSO- d_6 as the solvent at a concentration of 0.5 M. The spectral assignments are given in Table III (7).

Table III

Carbon-13 Magnetic Resonance Assignments for Cefoxitin

<u>ppm(δ)</u>	<u>Assignment</u>
170.4	amide C=O
162.5	carboxyl C=O
160.3	C ₈ , lactam C=O
156.3	carbamate C=O
136.5	C ₂ 1
126.5	C ₃ 1 and C ₄ 1
126.4	

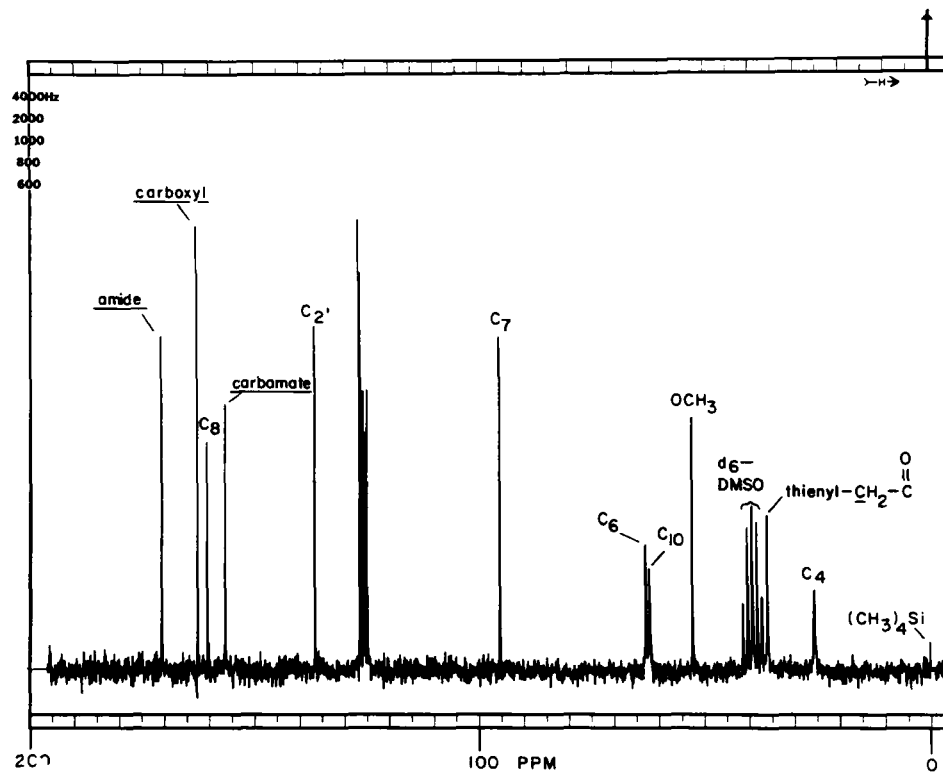


Figure 5. ^{13}C NMR Spectrum of Cefoxitin in DMSO-d_6 .

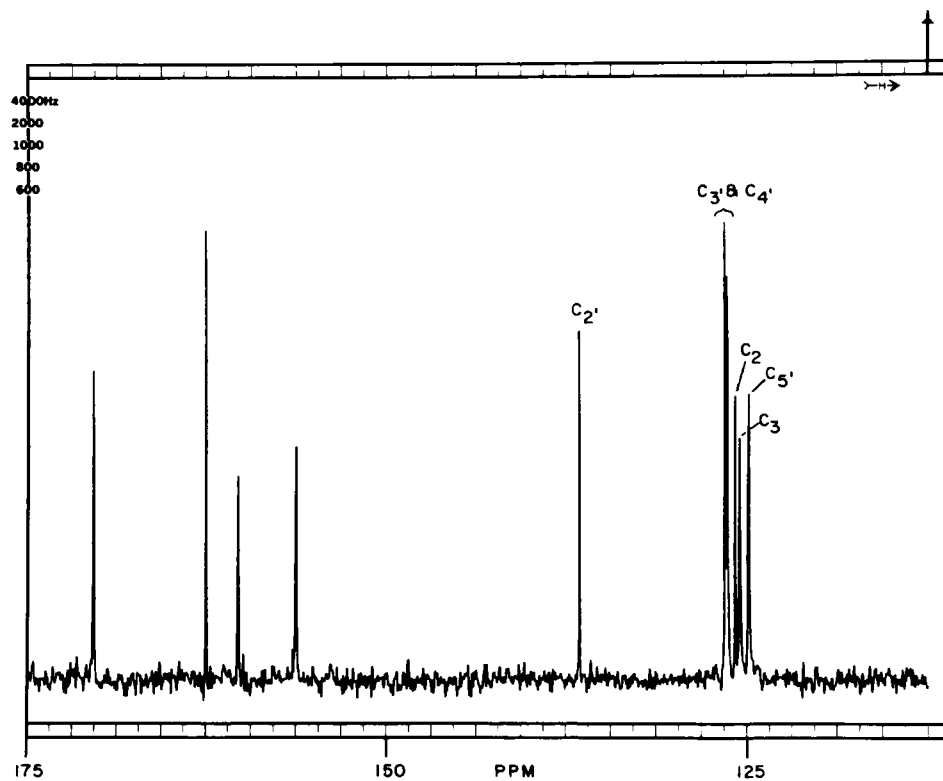


Figure 6. ^{13}C NMR Spectrum of Cefoxitin in DMSO-d_6 .

Table III (cont'd)

<u>ppm (δ)</u>	<u>Assignment</u>
125.8	C ₂
125.5	C ₃
124.9	C ₅ ¹
95.1	C ₇
62.8	C ₆
61.9	C ₁₀
52.5	OCH ₃
35.9	thienyl-CH ₂ -C(=O)-
25.7	C ₄

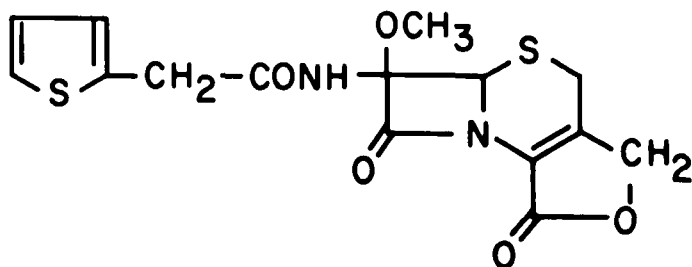
2.3 Ultraviolet Spectrum

The ultraviolet absorption spectrum of cefoxitin in pH 7.0 phosphate buffer is characterized by a peak at about 235 nm and a shoulder at about 262 nm (Figure 7) with $A_{1\%}^{1\text{cm}}$ values of approximately 330 and 209 respectively. The shoulder at 262 nm is due to the 3-cephem chromophore and the maximum at 235 nm is due to thienyl-acetyl side chain.

2.4 Mass Spectrum

The mass spectrum of cefoxitin was obtained using a Finnigan 3200 mass spectrometer in the electron impact mode with an ionizing energy of 70 eV and a probe temperature of 175°C. The output from the mass spectrometer was analyzed (8) and presented in the form of a bar graph shown in Figure 8.

The ion of highest mass seen in the spectrum of cefoxitin free acid (molecular weight 427) is m/e 366, which corresponds to the lactone formed from the



Cefoxitin lactone

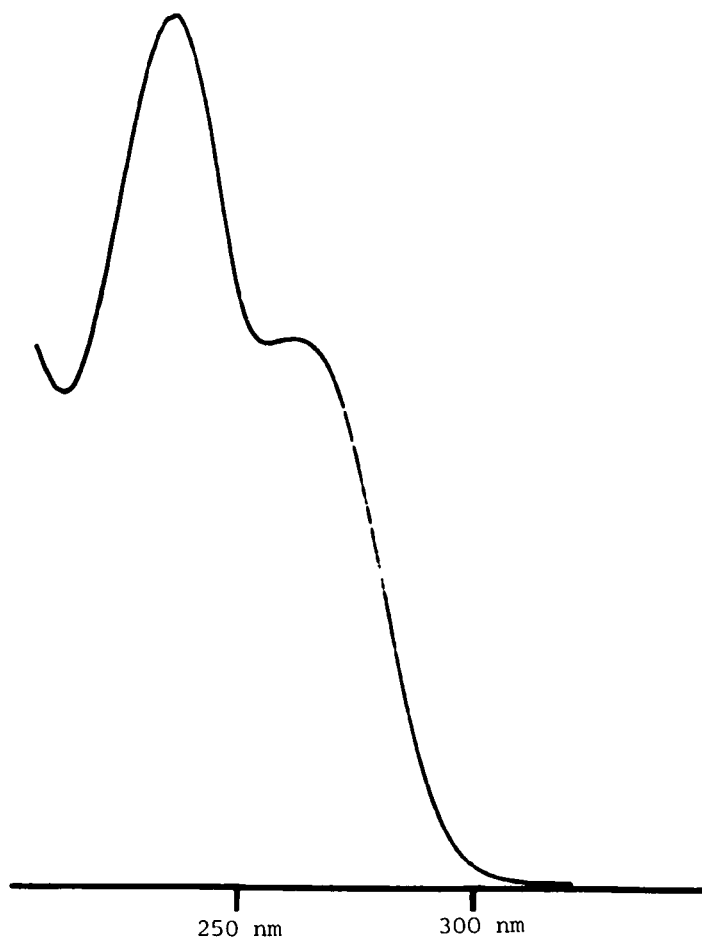


Figure 7. UV Spectrum of Cefoxitin in pH 7 Phosphate Buffer.
Concentration: 2.75 mg/100 ml.

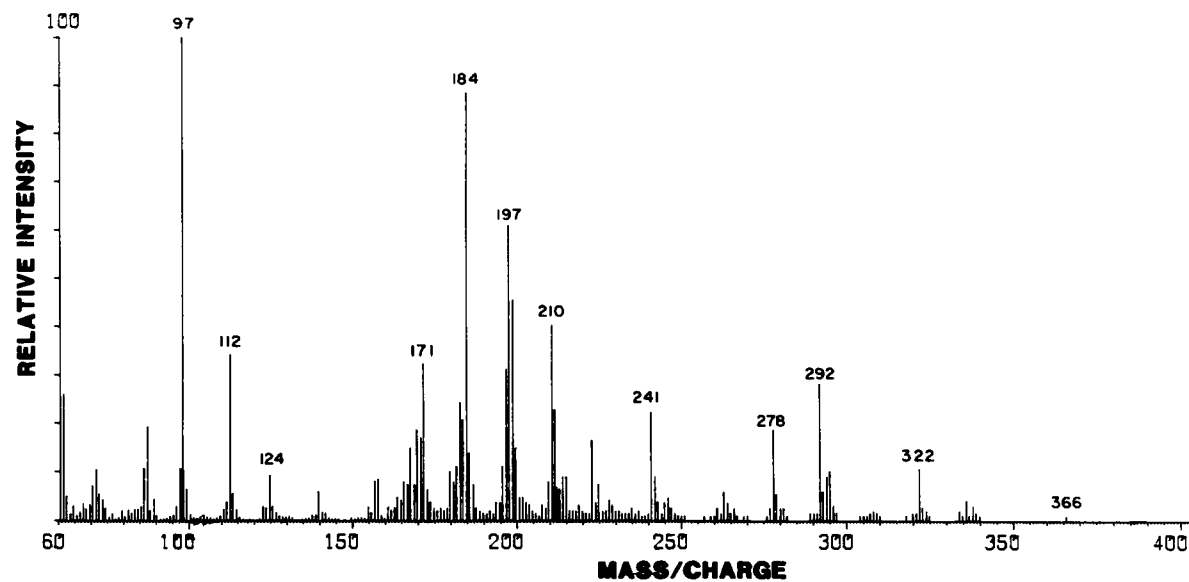
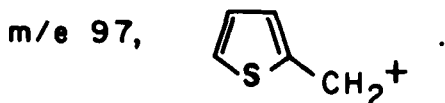
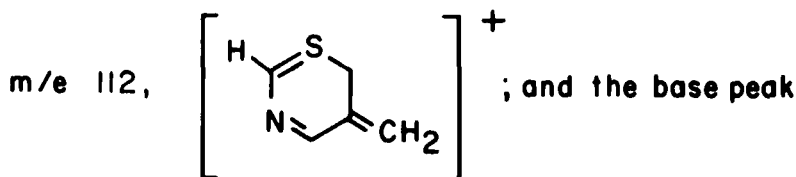
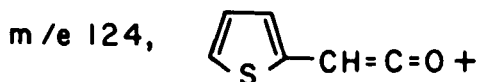


Figure 8. Low Resolution Mass Spectrum of Cefoxitin

carboxylic acid and carbamate group. The next ion (m/e 322) corresponds to loss of CO_2 from the lactone. Scission of the side chain amide bond of the lactone gives m/e 241, which loses CH_3O to give m/e 210. At lower mass one sees



2.5 Optical Rotation

The specific rotation of a 1% (w/v) sodium cefoxitin solution in methanol determined at 589 nm and 25°C is $+210^\circ \pm 4^\circ$ calculated on an anhydrous and solvent free basis.

2.6 Solubility

The solubility of sodium cefoxitin in the following solvents at room temperature is stated in terms of the U.S.P. definitions: very soluble in water, sparingly soluble in methanol and dimethylformamide, slightly soluble in ethanol and insoluble in ether, chloroform, acetone aromatic and aliphatic hydrocarbons.

The solubility of cefoxitin in water is typical of an organic acid with limited aqueous solubility and increases with increasing pH. At pH 1, the measured solubility is about 0.3 mg/ml and at pH 4, the observed value is about 25 mg/ml (9).

2.7 Crystal Properties

2.71 Crystallinity

Sodium cefoxitin exists in several solvated and in a desolvated crystalline form. An amorphous form has also been identified. X-ray powder diffraction patterns and polarized light microscopy can distinguish crystalline from amorphous forms (10).

2.72 X-Ray Powder Diffraction

The x-ray powder diffraction pattern of crystalline desolvated sodium cefoxitin was obtained with a Philips-Norelco diffractometer, using copper K α radiation. Samples of this form show broad diffraction lines suggesting that the solid is microcrystalline and composed of extremely small crystallites (11). The amorphous form shows no distinct X-ray powder diffraction pattern.

2.73 Differential Scanning Calorimetry

Crystalline sodium cefoxitin exhibits a decomposition exotherm at approximately 190 $^{\circ}$ -200 $^{\circ}$ C by differential scanning calorimetry (DSC). The amorphous form does not exhibit any well-defined thermal transitions by DSC below 350 $^{\circ}$ C. Gradual decomposition of this solid is observed by DSC as temperatures increase over 150 $^{\circ}$ C (11).

2.8 Hygroscopicity

The water uptake of sodium cefoxitin as a function of relative humidity was studied at ambient temperature (11). Equilibrium water levels were approximately 1-2% at 35% RH, 4-6% at 47% RH and 15-17% at 76% RH.

2.9 Acid Dissociation Constant

The dissociation constant of cefoxitin derived from aqueous titration (12) and solubility data (9) are in good agreement and indicate that cefoxitin is a relatively strong organic acid with a pKa of approximately 2.2.

3. Synthesis

Cefoxitin has been prepared by chemical modification of cephamycin C, a naturally occurring antibiotic produced by *Streptomyces lactamdurans* (13, 14). This route is presented in Figure 9. Cephamycin C (I) is tosylated to the N-tosyl derivative (II) and then esterified with methyl chloromethyl ether to yield the methoxymethyl ester (III). The ester is then treated with 2-thienylacetyl chloride to exchange the aminoadipoyl side chain for thienylacetyl. The ester protecting group is then removed with acid to yield cefoxitin (IV).

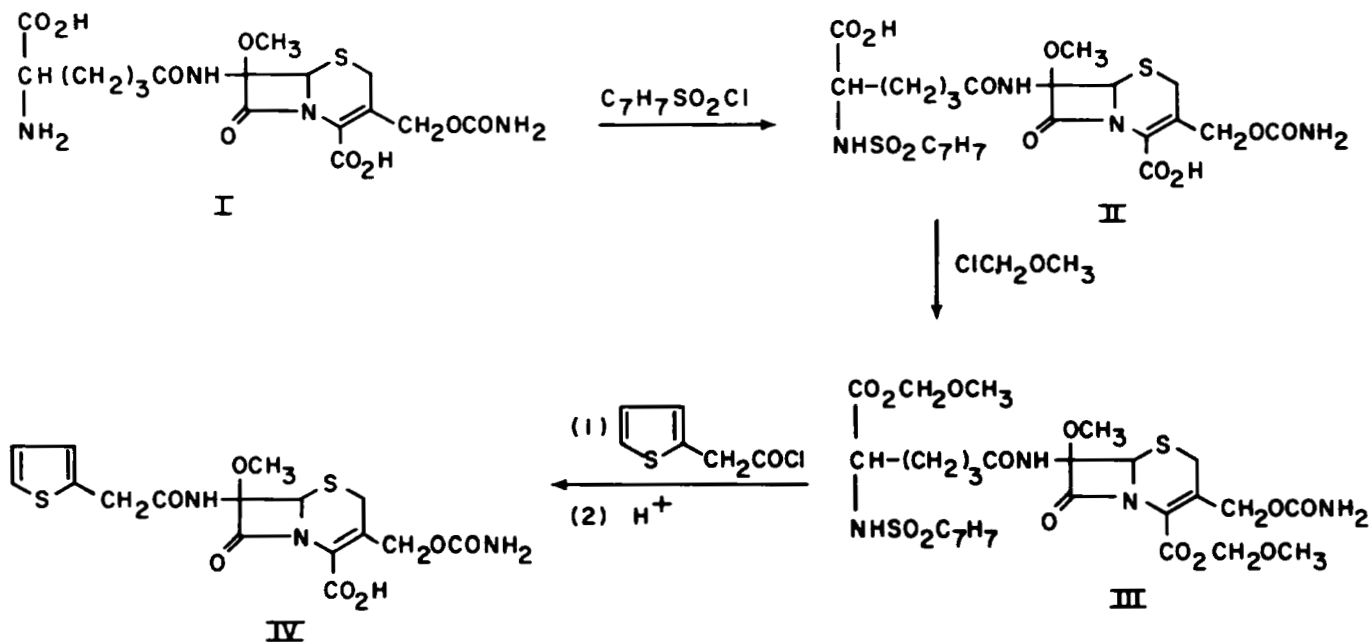


Figure 9
Synthesis of Cefoxitin from Cephameycin C

Cefoxitin has also been prepared by total synthesis (15) and via the acylation and methoxylation of 7-amino cephalosporanic acid (16, 17, 18, 19).

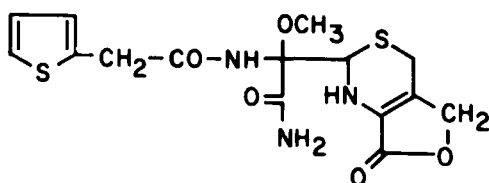
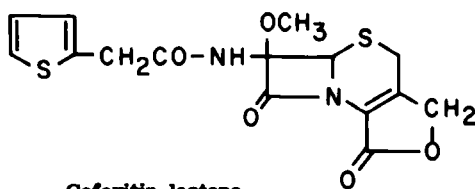
4. Stability-Degradation Products

4.1 Bulk Stability

At room temperature sodium cefoxitin is stable for at least three years when protected from moisture. At elevated temperature, the solid exhibits a biphasic decomposition pattern typified by an initial more rapid decomposition period followed by a slower decay period (20). This phenomenon may be related to degradation of cefoxitin by low levels of water in the sample. Since β -lactam cleavage is water-consuming, the extent of this degradation pathway is limited by available water in the solid. Amorphous sodium cefoxitin has been shown to be considerably less stable than its corresponding crystalline form (20).

A temperature dependent discoloration of the solid has been noted. The discoloration is negligible at 5°C and becomes greater at elevated temperature. It has been shown that an inert atmosphere (argon or nitrogen) markedly decreases this change. This development of color is not directly related to loss of potency, i.e. considerable discoloration can occur with no measurable loss of potency.

Several degradation products of solid sodium cefoxitin have been identified. From material stored at 60°C for three days, two compounds have been isolated and identified.



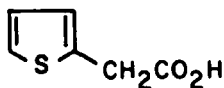
2-(methoxy)[2'-(thienylacetamido)]-carboxamidomethyl-5-hydroxymethyl-6H-2,3-dihydro-1,3-thiazine-4-carboxylic acid lactone

4.2 Solution Stability

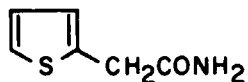
The solution stability of sodium cefoxitin has been studied in aqueous buffers in the pH range 3 to 9 (20). The degradation of sodium cefoxitin in this pH range follows apparent first-order kinetics. Maximum stability in water is in the pH range of 5-7. Under these pH conditions, sodium cefoxitin undergoes 10% chemical loss in two days at 25°C. Ten percent loss at pH 3 occurs in about 40 hours and at pH 9 in about 14 hours. TLC studies were carried out during kinetic runs. Patterns become complex suggesting that the initial β -lactam hydrolysis product is unstable and susceptible to transformation to a considerable number of secondary products (20).

The solution stability of sodium cefoxitin was also studied after constitution with frequently used I.V. infusions and admixture with commonly used I.V. and I.M. additives (21). Stability in these systems was essentially the same as that observed for unbuffered solutions. In these studies, sodium cefoxitin was shown to maintain potency in solution for at least 30 days at 5°C and for 30 weeks when stored in the frozen state.

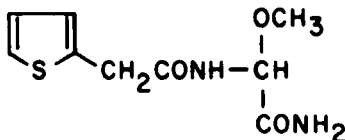
In an attempt to isolate degradation products formed in solution, a 10% aqueous solution of sodium cefoxitin was heated for four days at 80°C and then subjected to preparative TLC. Fractions isolated were examined by NMR, mass spectrometry and infrared. The following compounds were identified.



Thiophene-2-acetic acid



Thiophene-2-acetamide



N-(2'-methoxyacetamido) thiophene-2-acetamide

5. Pharmacokinetics and Metabolism

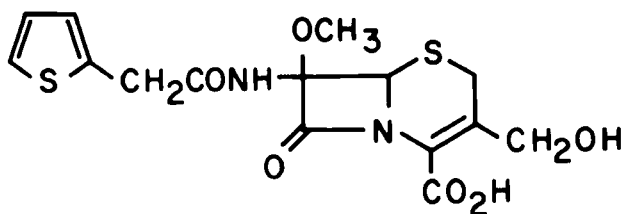
The pharmacokinetics and metabolism of sodium cefoxitin in humans following parenteral administration have been the subject of a number of investigations and reviews (22,31,32).

5.1 Pharmacokinetics

Following intravenous administration (bolus or infusion), cefoxitin is distributed rapidly between serum and tissue and exhibits a terminal serum half-life of 30 to 50 minutes. Total body clearance of cefoxitin ranges from approximately 250 ml to 350 ml/min while renal clearance is approximately 200 to 300 ml/min. Urine contains at least 90% of the dose as unchanged drug and less than 5% of the dose is eliminated by metabolism and biliary clearance (23,24,25). The disposition kinetics are first order, showing no effect of dose (0.25 to 3 g.) or infusion rate. Multiple dose regimens in this range given every four hours do not cause accumulation in healthy volunteers. The volume of distribution in the vascular compartment is about 8 liters (26-34). These data are adequately described by a two-compartment open model with elimination occurring from the central compartment.

5.2 Metabolism

Sodium cefoxitin is not metabolized appreciably in man. Urine samples from several human studies were separated by HPLC and TLC techniques. These studies show that more than 90% of the cefoxitin administered by either the intravenous or intramuscular route is recovered in the urine as intact drug. A microbiologically inactive metabolite, descarbamoyl cefoxitin, was found to the extent of 1-6% in some individuals, 2 to 4 hours post dosing (26,27). This metabolite was not found in the urine of all subjects.



Descarbamoyl cefoxitin

5.3 Intramuscular Absorption and Bioavailability

Sodium cefoxitin is rapidly and completely absorbed following intramuscular administration. Peak serum levels are attained in 30 minutes or less and 85 to 95% of the i.m. dose is recovered in urine within 12 hours of administration.

Intramuscular administration of sodium cefoxitin with either 0.5 or 1.0% lidocaine hydrochloride as a diluent has no apparent effect on the bioavailability of the antibiotic (32,35).

5.4 Effect of Probenecid

The concurrent oral or intravenous administration of probenecid with i.m. or i.v. injections of cefoxitin has a large influence on the time course of the antibiotic in serum (26,36). Probenecid administered intravenously concurrent with cefoxitin increases the serum half-life of cefoxitin from approximately 40 minutes to 80 minutes and reduces the renal clearance of the drug from 200-300 ml/min to less than 100 ml/min.

6. Methods of Analysis

6.1 Identification Tests

Ultraviolet spectrophotometry is used to identify sodium cefoxitin. The spectrum of a sample dissolved in pH 6.0 phosphate buffer scanned from 220 to 310 nm compares qualitatively to that of a cefoxitin standard similarly tested.

The identity of sodium cefoxitin is also established by infrared spectroscopy. The infrared absorbance spectrum of a solid sample prepared either as a potassium bromide disc or mineral oil mull is compared to a standard sample prepared in an identical manner.

A color test has been employed for the detection of cefoxitin in solution (37). To the residue obtained by drying a solution containing 10-50 mcg of cefoxitin is added 1-2 ml of 0.01% ninhydrin in concentrated sulfuric acid and the color is allowed to develop at room temperature. A vivid blue color appears in a few minutes. Other cephalosporin antibiotics do not give the same color response.

Cefoxitin and fifteen other cephalosporins have been identified by thin-layer chromatography coupled with color reactions (38) and by spectroscopic methods (39).

6.2 Ultraviolet Spectrophotometric Analysis

Intact sodium cefoxitin exhibits a UV absorption band near 262 nm attributed to the $O=C-N-C=C$ linkage in the molecule. Beta-lactam ring opening leads to disappearance of this absorption band. This observation is the basis of a quantitative assay for cefoxitin which is stability indicating. Calculation of intact compound is based on the net absorbance at 262 nm of the sample and of the standard in pH 6.0 phosphate buffer as determined by subtracting a base line correction at 262 nm from the maximum at the same wavelength. The correction is found by extending to 262 nm the straight portion of the UV curve between 340 and 300 nm.

6.3 Chromatographic Analysis

6.31 Thin Layer Chromatography

Thin layer chromatography using the system chloroform/acetone/formic acid (10:9:1) with 0.25 mm silica gel plates has been employed for both sodium cefoxitin and the free acid. The air dried plate is sprayed with 0.2% p-dimethylaminocinnamaldehyde in methanol/conc. sulfuric acid (4:1) and heated at 105° for five minutes. The R_f for cefoxitin in this system is approximately 0.45.

Cefoxitin has also been chromatographed on silica with developing solvents of n-butanol/water/acetic acid (4:1:1) and benzene/methanol/acetic acid (50:10:6) giving approximate R_f values of 0.7 and 0.2 respectively. Detection can be accomplished by fluorescence quenching or iodine staining.

6.32 High Performance Liquid Chromatography

Several HPLC procedures have been developed to separate cefoxitin from process impurities and degradates. A system frequently used is described below.

Mobile Phase: 20% acetonitrile in water containing 1% acetic acid.

Column: Ten micron, microporous octadecylsilane bonded reversed phase packing in a 3.9 mm X 30 cm column. Column temperature and pressure at 25°C (or ambient) and 1000-1500 psig, respectively. Flow rate of 1.0-1.3 ml/min.

Detection: U.V. at 254 nm

Procedure: Aliquots (10 mcl) of a sample solution containing 0.25 mg/ml in 0.02 M pH 7 phosphate buffer are injected. The retention time for cefoxitin is approximately 10-15 minutes.

6.4 Perchloric Acid Titration

Sodium cefoxitin can be determined by non-aqueous titration. An accurately weighed sample of about 800 mg is dissolved in about 100 ml of glacial acetic acid and titrated potentiometrically with standardized 0.1N perchloric acid in spectral grade dioxane. An electrode pair is employed consisting of a calomel electrode which has been refilled with 0.1N LiClO_4 in acetic anhydride as the indicating electrode, and a platinum ring as the reference electrode.

6.5 Iodometric Assay

Sodium cefoxitin bulk chemical and formulations can be determined by iodometric assay. In the assay, aliquots of the sample and of a suitable cefoxitin standard solution are hydrolyzed for 20 minutes with 1N NaOH and then acidified to pH 3.0 with acetate buffer and 1N HCl. Then, 0.01N iodine solution is added and, after a 20-minute wait, the excess iodine is titrated with 0.01N sodium thiosulfate solution to a starch end point. A blank determination is made at the same time on aliquots of both sample and standard solutions treated only with buffer and 0.01N iodine and allowed to stand for 20 minutes.

An automated iodometric assay has been developed based on the manual method described above with the exception that excess iodine is measured colorimetrically rather than by thiosulfate titration (40).

6.6 Microbiological Assay

For bulk and formulated products an agar diffusion (plate) assay can be conveniently carried out using Staphylococcus aureus as the test organism (41).

6.7 Hydroxylamine Assay

Sodium cefoxitin can be determined by means of the colored complex formed between ferric ion and the hydroxamic acid formed by the action of hydroxylamine on the beta-lactam (42). This method has been successfully automated

and has been specified by the FDA as the definitive assay method for certification of the antibiotic.

7. Determination in Biological Fluids

High performance liquid chromatography has been utilized for the determination of cefoxitin in biological fluids. Cefoxitin and its descarbamoyl metabolite have been quantitatively analyzed in human urine employing an anion-exchange column with U.V. detection (23). More recently Wheeler, et al. (43) have employed HPLC utilizing a C-18 reversed phase packing and a solvent system of acetonitrile/acetic acid/0.005M potassium dihydrogen phosphate (25/0.5/74.5, v/v/v) for the quantitation of cefoxitin in serum and saliva.

An HPLC method has been developed for the determination of cefoxitin in serum which lends itself to automation (44). The system is described below:

Mobile Phase: 11% methanol in pH 6.86 buffer containing 1% acetonitrile.

Column: Ten micron C-8 reversed phase packed in a 3.9 mm x 25 cm column with a disposable precolumn in line and a precolumn in the injection loop. A flow rate of 3.0 ml/min is used.

Detection: U.V. at 254 nm.

Procedure: Serum samples (100 μ l) are treated with 100 μ l of internal standard (aqueous cefmetazole, 15 μ l/ml and 75 μ l 10% trichloroacetic acid. Samples are mixed, allowed to stand for 15 minutes and centrifuged to remove precipitated protein. For automated analysis, clear supernatant is transferred to microcentrifuge tubes. A standard curve is generated by spiking blank serum with appropriate levels of cefoxitin and then processing as described above.

Cefoxitin in biological fluids has also been determined microbiologically by the cup-plate diffusion-technique using either *Staphylococcus aureus* MB2876 (26,27) or *Bacillus subtilis* MB36 (35) as the test organism.

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CLOFIBRATE

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1. Description1.1 Nomenclature1.11 Chemical Names

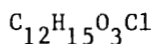
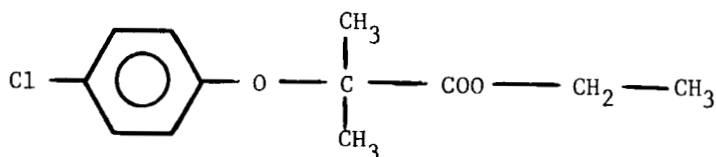
- a. 2-(4-chlorophenoxy)-2-methylpropanoic acid ethyl ester.
- b. Ethyl 2-p-chlorophenoxy isobutyrate
- c. Ethyl 2-(p-chlorophenoxy)-2-methyl propionate.
- d. Propanoic acid, 2-(4-chlorophenoxy)-2-methyl-, ethyl ester.

1.12 Generic Name

Clofibrate

1.13 Trade Names

Amotril ; Anparton ; Ateculon ; Atheropront ;
 Ateriosan ; Atromidin ; Atromid-s ; Claripex ;
 Clobren-SF ; CPIB ; Hyclorate ; Lipavion ;
 Neo-Atromid ; Normet ; Recolip ; Regelan ;
 Serotinx ; Skleromex (1).

1.2 Formulae1.21 Empirical1.22 Structural

1.23 CAS Registry No.

(637-07-0)

1.24 Wiswesser Line Notation (2)

GR DOXVO 2

1.3 Molecular Weight

242.71

1.4 Appearance, Color, Taste, Odor

Stable, colorless to pale-yellow liquid with a faint, characteristic odor and a characteristic taste.

2. Physical Properties2.1 Boiling Range

158-160

148-150²⁰2.2 Density d^{25} : 1.138 - 1.1442.3 Refractive Index n_D^{20} 1.500 - 1.5052.4 Solubility

Insoluble in water, soluble in acetone, alcohol, benzene, and chloroform.

2.5 Spectral Properties2.51 Infrared Spectrum

The Infrared Spectrum of Clofibrate is recorded as a film on a Unicam Sp-1025 Spectrophotometer and is shown in Fig.1. The assignments for the characteristic bands in the Infrared Spectrum are listed in Table 1.

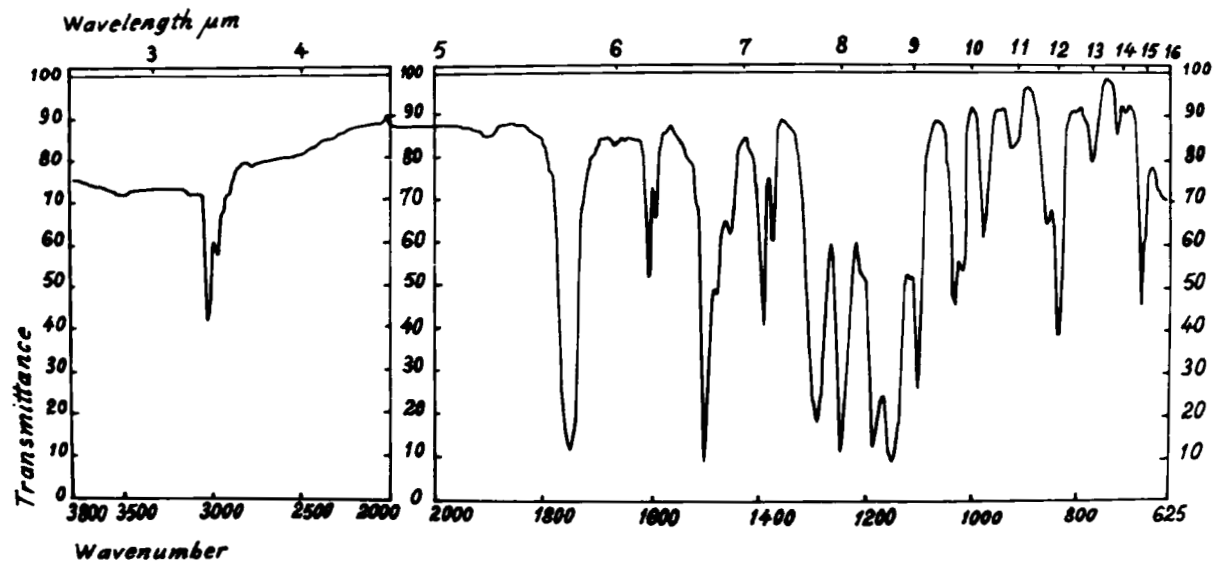


Fig. 1. IR Spectrum of Clofibrate as Film.

Table I

IR Characteristics of Clofibrate

<u>Frequency Cm^{-1}</u>	<u>Assignments (3)</u>
1750	C = O (ester).
1605	C = C (aromatic).
1590	
1500	
1150	C-O-C
1100	(C-O stretching).
1390	$\text{C}(\text{CH}_3)_2$
1375	
	(Symmetrical deformation).
830	aromatic para-disubstitution
770	C-Cl
680	

Other bands characteristic of Clofibrate are 3020, 2970, 1290, 1250, 1190, 1030, 1020, 980 and 920 Cm^{-1} .

2.52 Ultraviolet Spectrum (UV)

The UV spectrum of clofibrate in methanol was scanned from 400–200 nm using Varian Cary 219 Spectrophotometer and is shown in Fig.2. The spectrum exhibits three maxima at 288 (708), 280 (10233) and 226 (8511).

2.53 Nuclear Magnetic Resonance Spectrum2.531 Proton Spectrum

The proton NMR Spectra of Clofibrate in deuterated chloroform and in Acetone- D_6 were recorded on a Varian T-60 A, 60 MHz NMR Spectrometer, using tetramethylsilane

as an internal reference. The PMR spectrum in deuterated chloroform is shown in Fig. 3. The PMR spectral assignment of clofibrate are given in Table 2 (4).

Table 2

PMR Characteristics of Clofibrate

Protons	Chemical Shifts		
	COCl_3	Acetone- D_6	
$-\text{CH}_2\text{CH}_3$	1.20	1.23	t
$-\text{C}-(\text{CH}_3)_2$	1.56	1.57	s
$-\text{CH}_2\text{CH}_3$	4.18	4.20	q
Four aromatic protons	6.96	7.03	q

s = singlet; t = triplet; q = quartet

2.532 ^{13}C -NMR Spectrum

^{13}C NMR Spectrum of clofibrate in deuterated chloroform using tetramethylsilane as an internal reference was recorded on a Jeol FX 100, 100 MHz instrument at ambient temperature and using 10 mm sample tube. The data consist of 8192 data points over a 5000 Hz Spectral Width. The completely decoupled spectrum is shown in Fig. 4. The carbon chemical shift values, shown in Table 3 are derived from both additivity principles and the off-Resonance Spectrum Fig. 5 (5).

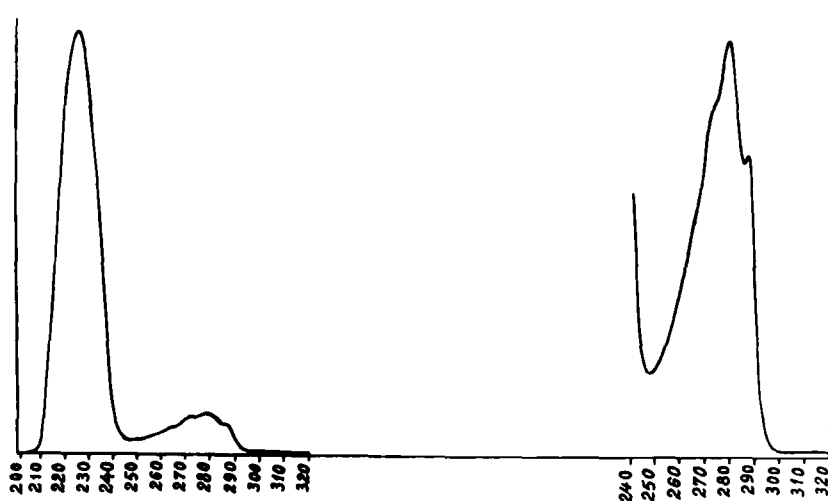


Fig. 2. UV Spectrum of Clofibrate in Methanol.

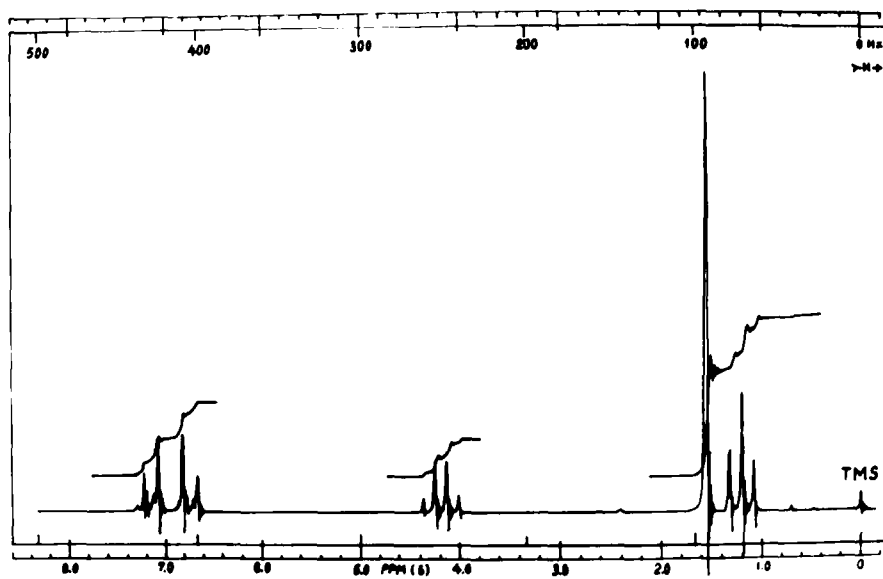


Fig. 3. PMR Spectrum of Clofibrate and Tetramethylsilane in CDCl_3

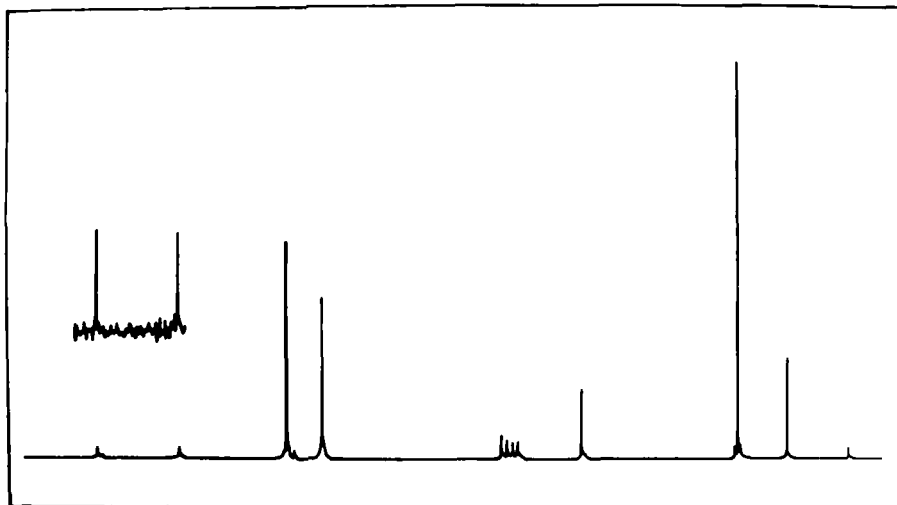


Fig. 4. ^{13}C -NMR Spectrum of Clofibrate in CDCl_3

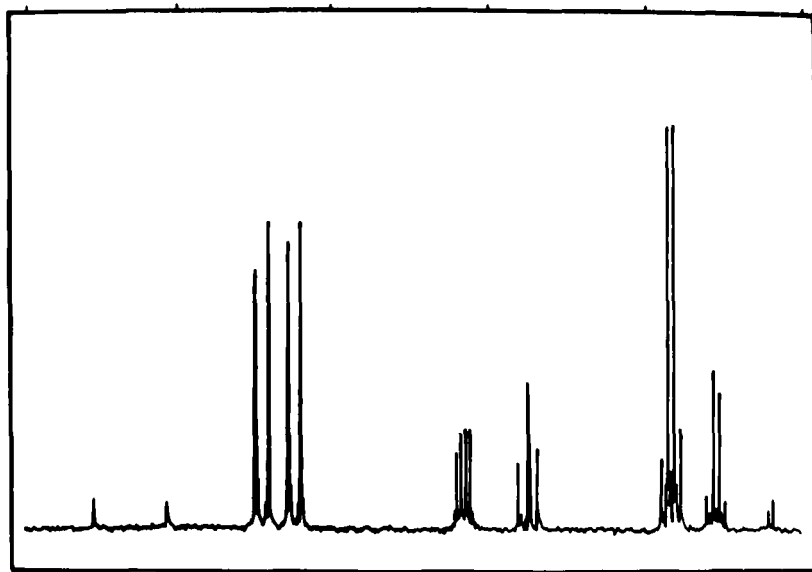


Fig. 5. ^{13}C -NMR Proton-Coupled Spectrum of Clofibrate in CDCl_3

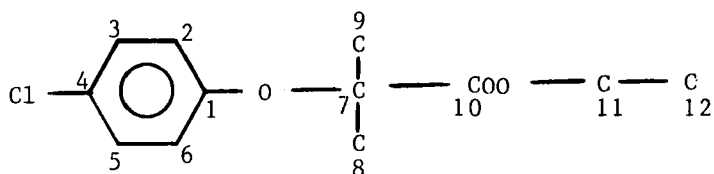


Table 3

¹³C NMR Characteristics of Clofibrate

Carbon No.	Chemical Shift ppm	Carbon No.	Chemical Shift ppm
1	154.18	7	79.62
2	120.85	8	25.34
3	129.04	9	25.34
4	121.43	10	173.67
5	129.04	11	61.30
6	120.85	12	14.03

2.54 Mass Spectrum

The mass spectrum of clofibrate (Fig. 6) obtained by electron impact ionization shows a molecular ion M^+ at m/e 242 (relative intensity 41.1%) and a base peak at 128. The proposed fragmentation pattern is presented in Table 4.

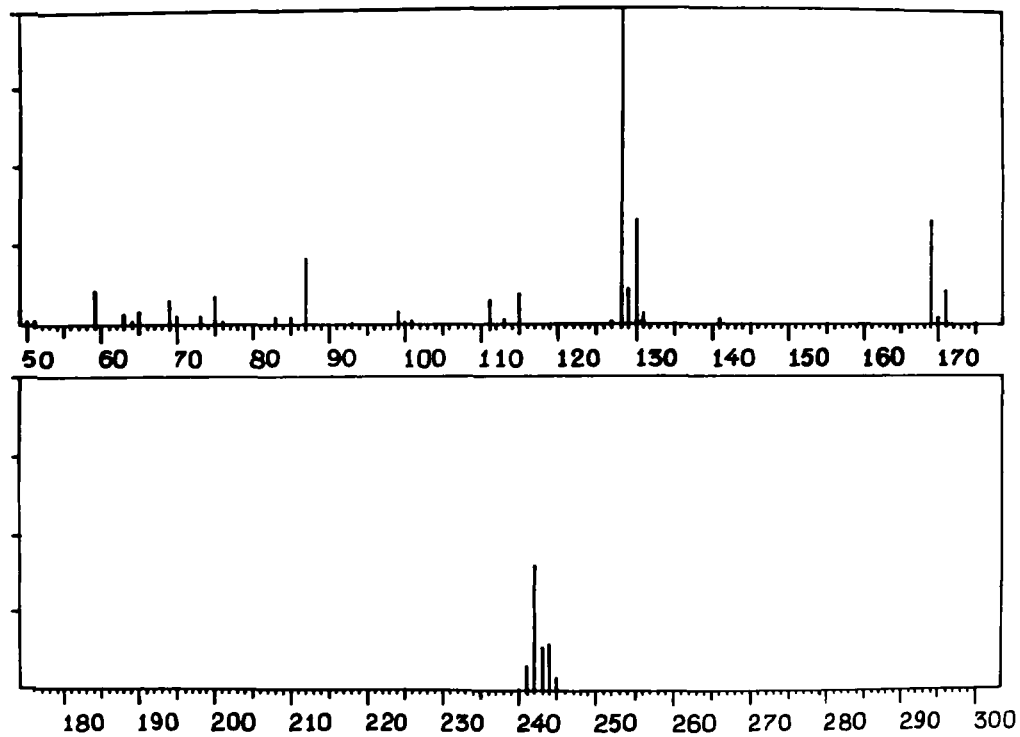
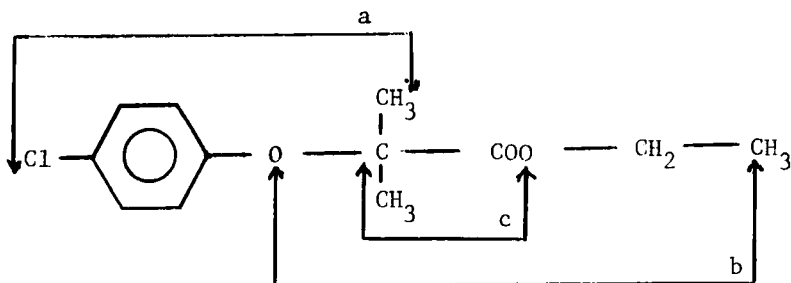


Fig. 6. Mass Spectrum of Clofibrate.

Table 4

Mass Fragmentation Pattern of Clofibrate.

<u>CI (Methane)</u>			<u>EI</u>		
<u>m/e</u>	<u>RI%</u>	<u>Ion</u>	<u>m/e</u>	<u>RI%</u>	<u>Ion</u>
243	100	M.H ⁺	242	41.1	M ⁺
169	27	a	169	33.1	a
129	10	-	130	33.0	b+2H
115	78	-	128	100.0	b
			87	21.2	c+H

RI = Relative Intensity

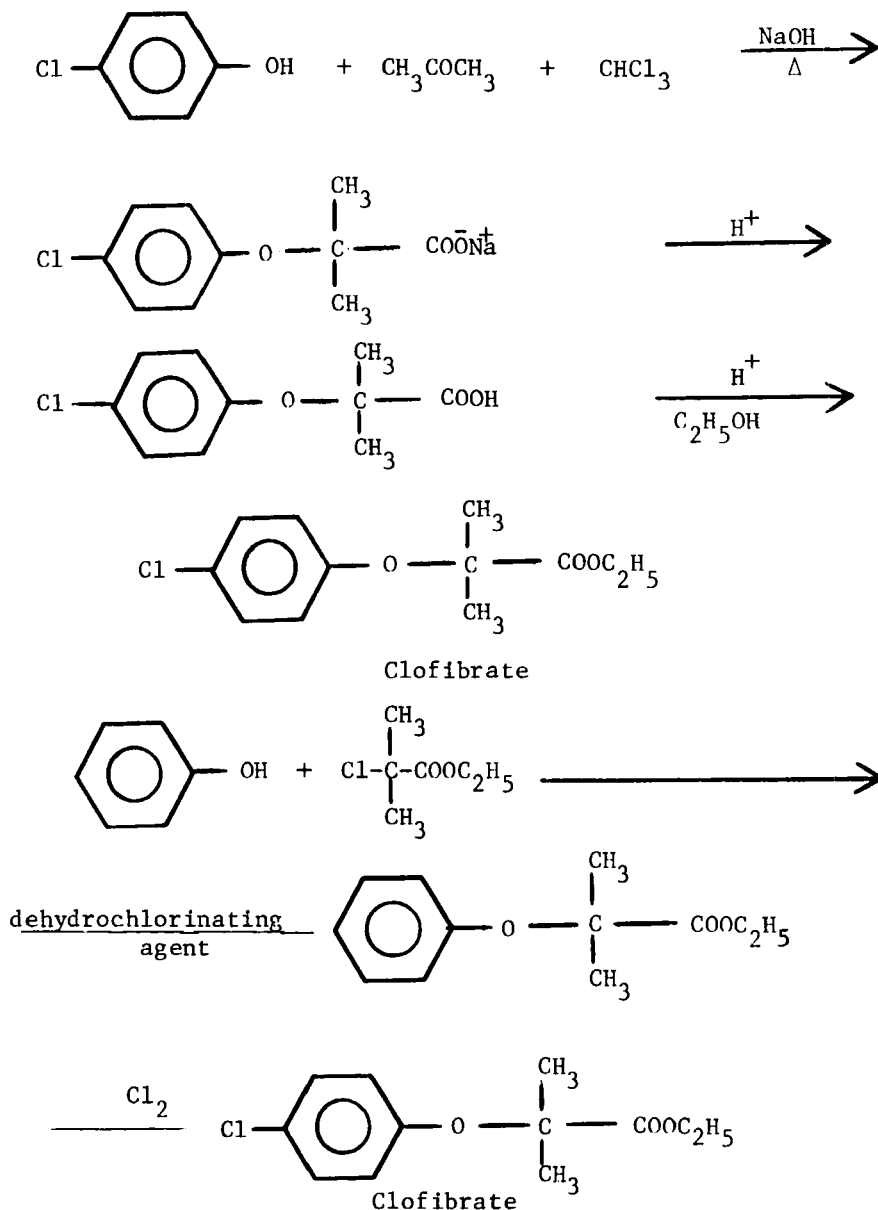
Mass spectral data, both by electron impact and by methane chemical ionization have also been reported.(2, 6, 7).

3. Synthesis:

The synthesis of Clofibrate has been achieved by two main methods (8-15).

Method I : This involves condensation of para-chlorophenol with acetone and chloroform in the presence of sodium hydroxide followed by esterification of the resulting acid, afforded clofibrate.

Method II : By condensing phenol with ethyl 2-chloro-2-methyl-propionate in the presence of a suitable dehydrochlorinating agent and then chlorination of the resultant product, afforded clofibrate.



4. Metabolism:

The metabolism of clofibrate and different clofibric acid derivatives in several animal species and in man were reported (16-22). It has been shown that clofibrate is completely hydrolysed to clofibric acid (para-chlorophenoxy isobutyric acid) which is then conjugated and excreted. In man the two conjugates of clofibric acid found in urine were present in plasma. The highest plasma concentrations of clofibric acid metabolites were generally found in patients with renal disease. Since clofibrate undergoes rapid hydrolysis in vivo and in vitro, the resulting clofibric acid is presumed to be the active drug. Studies in vivo and in vitro with clofibrate and clofibric acid indicate that the latter may exert its effect by multiple modes of action (23).

5. Pharmacology: (24-32)

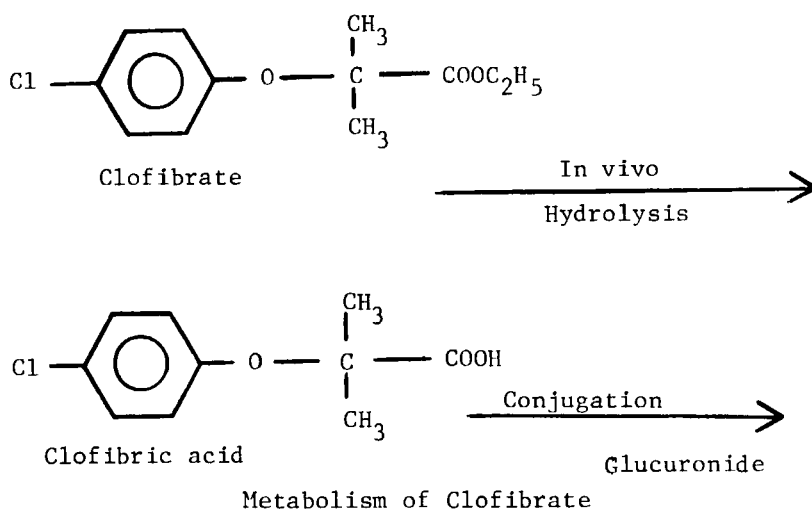
Clofibrate reduces elevated triglyceride and cholesterol concentrations in serum; the effect on serum lipoproteins is particularly evident on the very low-density fraction. When administered to rats, it caused a decrease in serum triglyceride and alterations in adipose tissue uptake and release of Lipids (33). The biochemical changes produced include a decrease in adenyl cyclase activity, inhibition of acetyl-coenzyme. A carboxylase, inhibition of cholesterol and triglyceride biosynthesis. The inhibition of hepatic triglyceride formation is an early metabolic consequence of clofibrate administration and precedes the fall in serum triglyceride. Its effects on blood coagulability suggest that it may reduce the hypercoagulability frequently associated with atherosclerosis. Uric acid concentrations, where elevated, have frequently shown a transient reduction, and the shortening of the recalcified Clotting-time, which occurs during post-prandial lipaemia, is prevented.

Clofibrate was first used in conjunction with androsterone but it became evident that the effects produced were not enhanced by the steroid. It is used in atherosclerotic conditions manifested in coronary heart disease and in cerebral and vascular diseases, in familial hypercholesterolemia and in xanthomatous conditions. Exudative diabetic retinopathy has been improved by clofibrate.

Samuel et al (34) reported the significant reduction of cholesterol levels by the combined oral administration of neomycin and clofibrate. Musa et al (35) studied the effects of clofibrate upon the distribution, metabolism,

transport and plasma binding of ^{131}I -thyroxine in euthyroid individuals. It had no consistent effect upon the binding capacities of thyroxine-binding globulin or thyroxine binding pre-albumin. The hepatic distribution space and content of thyroxine-iodine were lower after clofibrate. The hepatic thyroxine clearance and plasma-to-liver thyroxine flux were unchanged. Clofibrate did not alter the plasma thyroxine iodine, the daily thyroxine degradation rate or the total thyroxine distribution space. These findings failed to support the hypothesis that clofibrate produces its hypolipidemic effect by displacing thyroxine from its binding proteins and shunting it into the liver.

Harrison and Harden (36) studied the effect of clofibrate on 6 patients with hypothyroidism and ischemic heart disease. The patients were maintained on the maximum dose of thyroxine which they could tolerate but all still had evidence of hypothyroidism and levels of cholesterol in the serum were elevated. Clofibrate produced a rapid fall in serum cholesterol averaging 37% and was maintained up to the two years the drug was continued. In three patients it was possible to increase the dose of thyroxine during therapy with clofibrate; in two patients with untreated severe hypothyroidism, clofibrate was without effect on serum cholesterol until thyroxine was added.



6. Methods of Analysis

6.1 Elemental Analysis

C, 59.38% ; H, 6.23% ;
Cl, 14.61% ; O, 19.78%.

6.2 Identification Tests

Those mentioned in the B.P. (1980) (37).

- A. The infra-red absorption spectrum, Appendix II A, is concordant with the reference spectrum of clofibrate.
- B. The light absorption, in the range 220 to 250 nm, of a 2-cm layer of a 0.001 per cent w/v solution in absolute ethanol exhibits a maximum only at 226 nm; absorbance at 226 nm, about 0.91, Appendix II B.
- C. The light absorption, in the range 250 to 350 nm, of a 2-cm layer of a 0.01 per cent w/v solution, in absolute ethanol exhibits two maxima, at 280 nm; and 288 nm absorbance at 280 nm, about 0.87, and at 288 nm, about 0.62, Appendix II B.
- D. To 0.05 ml of a 10 per cent w/v solution in ether add 0.05 ml of a saturated solution of hydroxylammonium chloride in ethanol (96 per cent) and 0.05 ml of a saturated solution of potassium hydroxide in ethanol (96 per cent). Heat for two minutes on a water-bath, cool, acidify with 0.5 M hydrochloric acid, and add 0.05 ml of a 1 per cent w/v solution of iron (III) chloride; a violet colour is produced.

6.3 Purity Tests

- (a) Water Content : not more than 0.2%.
- (b) Acidity: Mix 10.0 g with 100 ml of neutralized

alcohol, add 3 drops of phenolphthalein T.S. and titrate with 0.1 N sodium hydroxide : not more than 0.9 ml is required for neutralization.

(c) Para-Chlorophenol:

The USP (XX) describes a spectrophotometric method for the detection of p-chlorophenol. The percentage of p-chlorophenol is not allowed to exceed 0.003%, while the B.P. (1980) describes a gas chromatographic procedure.

6.4 Official Methods: U.S.P. XX (38)

To a beaker containing 75 ml of 1 N sodium hydroxide add about 3 g of a strongly basic polystyrene anion-exchange resin, and allow the mixture to stand for about 15 minutes, with occasional stirring. Wash the resin with water until the last washing is neutral to litmus paper, then wash with three 50 ml portions of methanol. Place a plug of glass wool in the base of a 1-X 15 cm ion-exchange tube, and transfer to the tube a sufficient amount of Ion-exchange resin, slurried in methanol, to produce a column bed height of from 6-cm to 8-cm.

Transfer about 200 mg of Clofibrate, accurately weighed, to a 100 ml volumetric flask, add methanol to volume, and mix. Transfer 10.0 ml of this solution to the Ion-exchange column, and collect the eluate in a 100 ml volumetric flask. Rinse the column with 25 ml of methanol, collect the rinsing in the volumetric flask, dilute with methanol to volume, and mix. Transfer 5.0 ml of this solution to a 50 ml volumetric flask, dilute with methanol to volume, and mix.

Dissolve an accurately weighed quantity of USP Clofibrate RS in methanol, and dilute quantitatively and stepwise with methanol to obtain a solution having a known concentration of about 20 μg per ml.

Concomitantly determine the absorbances of the Standard preparation and the Assay prepara-

tion in 1-cm cells at the wavelength of maximum absorbance at about 226 nm, with a suitable spectrophotometer, using methanol as the blank. Calculate the quantity, in mg, of $C_{12}H_{15}ClO_3$ in the portion of Clofibrate taken by the formula $10C(A_U/A_S)$, in which C is the concentration, in μg per ml, of USP Clofibrate RS in the Standard preparation, and A_U and A_S are the absorbances of the Assay preparation and the Standard preparation, respectively.

6.5 Ultraviolet Spectrophotometry

Aftalion et al (39) reported that ethanol could not be used as a solvent for the ultraviolet spectrophotometry of clofibrate in gelatinous capsules with vegetable oils because the latter interfere strongly at the λ_{max} of clofibrate (227 nm). However, by using dioxane, the absorption coefficient of vegetable oil was found constant from 255 to 280 nm while that of clofibrate increased from 0.24 at 265 nm to 0.48 at 280 nm (using a 0.01% solution). In the assay, the content of 5 capsules was homogenized, 0.5 g dissolved in dioxane to give 25 ml solution, 1 ml diluted further with dioxane to 100 ml, and the absorption was determined at 280 nm and 265 nm with respect to a standard solution of 0.01 g vegetable oil in 100 ml dioxane measured at 280 nm. The true absorption was taken as twice the difference at the two wave lengths. Errors of $\pm 4\%$ were obtained when applied to synthetic mixture of 1:1.

A spectrophotometric assay had been described (40) for the quantitation of clofibric acid in plasma and urine. This involves solvent extraction of clofibric acid from acidified plasma or urine and subsequent measurement of the UV absorbance at 226 nm.

The U.S.P. XIX (41) describes an assay procedure for clofibrate based on the passage of the solution of clofibrate in methanol on a column of strongly basic polystyrene anion-exchange resin. The absorbances of the assay preparation and a standard clofibrate preparation are concomitantly determined in 1-cm cells at 226 nm using methanol as the blank.

6.6 Thin Layer Chromatography (42)

Mixtures of clofibrate and xanthinol nicotinate were separated on silica gel layers. Using chloroform as solvent, the R_f of clofibrate and xanthinol nicotinate are 82 and 3 respectively. Using chloroform acetic acid 95:5, the R_f values are: Clofibrate (78), xanthinol (0) and nicotinic acid (15).

6.7 Thin Layer - Gas Liquid Chromatography:

A specific and sensitive method for the determination of clofibrate in biological fluids was described (43). Clofibrate was separated from associated fatty acids by TLC and the methyl ester was quantified by GLC.

6.8 Gas Liquid Chromatography:

Gas liquid chromatographic methods occupy a prominent position in the quantitation of clofibrate in drugs, tissues and biological fluids. In the method of Silvestri (44), clofibrate is extracted from tablets with ethyl ether and from tissues, blood or urine by homogenization with the addition of NaCl and 7% $HClO_4$ solution with ether - light petroleum (1:1). The ether extract is washed with water, dried over anhydrous sodium sulphate and evaporated to small volume in a stream of nitrogen. GLC performed at $190^\circ C$ on a column (6 ft. x 1/8 in.) of 5% butane-diol succinate on Chromsorb P(100 to 120 mesh), with nitrogen as carrier gas and a flame ionization detector, methyl heptadecanoate is used as internal standard. Clofibric acid is similarly determined after adsorption on Amberlite IRA-400 and esterification by treatment with methanolic HCl . Overall recoveries were 98% ($\pm 5\%$) for the ester and 92% ($\pm 10\%$) for the free acid. The limit of detection is 0.5 μg of the ester or acid per ml of urine or g. of tissue.

Karmen and Haut (45) described a method for assaying clofibrate in serum based on GLC of the methyl ester. Two internal standards similar in chemical structure to clofibrate (chlorophenoxy acetic and chlorophenoxy propionic acids) are added; the compounds are extracted, converted to methyl esters and subjected to GLC using an alkali flame ionization

detector selectively sensitive to halogen.

Knuechel and Ochs (46) described a gas chromatographic method for the detection of clofibrate in the serum of treated patients. The retention time of clofibrate was found between methyl palmitate and methyl stearate. Clofibrate was detected in very small amounts in serum globulins which were separated by fractional precipitation, whereas the amounts extracted from albumin correlated well with those of the serum. Fractions of cholesterol ester from serum of clofibrate-treated patients, contained small amounts of clofibrate in spite of repeated fractionation on silica gel column, due to esterification of clofibrate by cholesterol.

Berlin (47) reported a quantitative gas chromatographic method of clofibric acid in plasma using 2-(4-chloro-3-methylphenoxy)-2-methylpropionic acid as internal standard. To blood plasma (200 μ l) 1s added H_3PO_4 acid 2M (50 μ l) and internal standard solution in toluene (0.5 ml). After shaking (10 min) the organic phase is transferred to a 3 ml tapered glasstube and the plasma is re-extracted with 0.5 ml toluene. Disodium hydrogen phosphate 0.5M (0.5 ml) is added to the combined organic phases and the mixture is shaken for 10 min. The organic phase is removed and the aqueous phase is made acidic with H_3PO_4 acid 5M (50 μ l) CH_2Cl_2 (100 μ l) is added and the mixture is extracted on a whirl-mixer for 30 seconds. After removal of the aqueous phase, diazomethane in ether (50 μ l) is added and the solution is evaporated under nitrogen to about 1/5 of its volume and one μ l is injected. Blank plasma samples showed no peaks interfering with the clofibric acid or internal standard methyl ester peaks. The method is limited to determination of plasma concentrations down to 1 μ g ml^{-1} due to the concentration chosen for the internal standard.

Another method for the quantitative determination of clofibric acid in blood plasma had been described (48). The substance is extracted from acidified plasma into benzene, the extract is evaporated to dryness and the residue is methylated and submitted to chromatography on a glass column packed with 3% OV-17 on chromosorb WHP, 100-120 mesh. The conditions are as follows: injector temperature, 180°C; detector

temperature, 210^o; column initial temperature, 150^o for 6 min. then programmed at 195^o at 10^o/min. and held at the final temperature for 5 min. before re-cycle. The flow rates were: helium carrier gas, 25 ml/min. helium auxiliary gas, 35 ml/min; air, 400 ml/min. hydrogen, 40 ml/min. The retention times of clofibric acid methyl ester and of actadecane (external standard) were 3 and 5 min. respectively.

A rapid gas chromatographic method is described (49) for the determination of clofibric acid in plasma and urine. The assay involves an extraction into toluene and back-extraction of clofibric acid and the internal standard (2-naphthoic acid) into the methylating agent (trimethylanilinium hydroxide). The silanized glass column (6 ft. x 4 mm i.d.) was packed with 3% of SE-30 on 80-100 mesh Gas Chrom Q and was operated at 150^oC with a carrier gas (nitrogen) flow-rate of 25 ml/min; the injection port temperature was 290^oC. The flame ionization detector was operated at 270^oC with a hydrogen flow-rate of 20 ml/min. and an oxygen flow-rate of 200 ml/min. Under these conditions, the retention times were 1.5 min. for clofibric acid and 2.5 min. for the internal standard. The blood samples are drawn into heparinized tubes and the plasma was separated by centrifugation. To 1.0 ml of plasma in a 15 ml glass tube were added 1 ml of 0.4 M hydrochloric acid and 6 ml of toluene containing 120 µg of the internal standard. The tube was shaken for 5 min. and centrifuged for 3 min. at 4000 g. A 5 ml portion of the organic phase is transferred to a pointed centrifuge tube. Trimethylanilinium hydroxide (50 µl) is added and the mixture is extracted on a Vortex-mixer for 1 min. After brief centrifugation, 1 µl of the aqueous layer is injected directly. The determination of urinary clofibric acid is carried out essentially as described for plasma. For analysis of glucuronide metabolite of clofibric acid in urine, the sample is diluted 1:10 with 0.2 M sodium acetate buffer of pH 5.0 and 4 ml of the diluted sample are incubated overnight with 2000 Fishman units of a glucuronidase-aryl-sulphatase preparation from *Helix Pomatia*.

Wolf and Zimmerman (50) described a simultaneous GLC determination of Clofibrate and its metabolite clofibric acid in human plasma. This has been achieved by using a gas chromatographic column packed

with Gas Chrom Q coated with 10% Silar 10 C and nitrogen as carrier gas. The method is rapid and do not require a derivatization step, it is sensitive to 1 $\mu\text{g/ml}$ of either compound in biological samples and could be used to characterize the invivo conversion of clofibrate ester to the free acid.

A comparative study of gas-liquid chromatographic behaviour of the pentafluorobenzyl esters and the methyl esters of ten chlorophenoxyalkyl acids including clofibric acid was also reported (51). Para-chlorophenol and para-hydroxy benzoic acid esters which are added as capsule preservatives were determined by gas chromatography (52). These were reacted with $(\text{EtO})_2\text{P(O)Cl}$ and MeONa in hexane at 50° for 30 min. to give the diethylphosphate esters. These esters and clofibrate were separated and determined on a column packed with 3.5% silicone JXR on Chromosorb G and HP at 190° under N_2 carrier. Flame photometric and Flame thermionic detectors were used. The coefficients of variation for 3.87, 7.74 μg para-chlorophenol in 10 ml were 0.87, 0.56% with FTB and 1.5, 0.56% with FPD respectively. Parachlorophenol in commercial preparations was 1.6-5.1 ppm.

6.9 Gas Chromatography - Mass Spectrometry:

Impurities in clofibrate have been studied GC-MS (53). Three main impurities were found, the methyl ester analogue of clofibrate, its deschloroanalogue and the dichloroanalogue.

Johansson and Ryhage (54) have identified other impurities in three clofibrate preparations. Samples were obtained by dissolving 0.5 ml of capsule - content in 0.5 ml chloroform 5 μl of the sample was injected into the combined gas chromatograph - mass spectrometer LKB 2091. The G.C. column used was 3% SE-30 glass column 2.7 M x 2 mm (i.d.). A constant temperature of 160°C for the first 8 min. was used. The carrier gas flow-rate was 25 ml helium/min. The mass spectra were obtained with a constant accelerating voltage of 3.5 KV, an electron energy of 70 eV and an ionizing current of 100 μA . Repetitive scanning of the mass range 10 to 500 in 25 was used.

6.10 High-Performance Liquid Chromatography:

Bjornsson et al (55) developed a rapid, sensitive and specific high pressure liquid chromatographic method for the quantitative analysis of clofibric acid in plasma, saliva and urine. Plasma (0.1 - 1 ml), saliva (1 ml) or urine diluted 1:100 with distilled water (1.0 ml) is placed in a screw-capped tube, and 100 μ l of internal standard solution (containing 6.7 μ g of the internal standard), 0.5 ml of 0.5 N sulphuric acid and 5 ml of toluene are added. The samples are extracted by mixing for 10 min. followed by centrifugation at 1200 g for 10 min. to separate the organic and aqueous phases. The lower aqueous phase is frozen by immersing the tube in a dry-ice acetone bath, and the organic phase is poured into another tube, which has an elongated cone at its base. Then 50 μ l of 0.2 N NaOH are added, and the mixture is extracted on a Vortex-mixer for 2 min. After brief centrifugation, the aqueous phase is drawn into a syringe that already contains 10 μ l of a solution of 5% glacial acetic acid in water and this mixture is injected into the chromatograph. For the analysis of the glucuronide conjugate of clofibric acid in urine, 5 ml of 6N hydrochloric acid were added to each sample, and the solutions were heated at 98°C for 30 min. before the extraction. The samples were then cooled and analysed as described above, except that the addition of dilute sulphuric acid in the first step was omitted. A Varian Micropak CH-10 reverse-phase column (25 cm x 6.3 mm o.d. x 2.2 mm i.d.) was used. The absorbance was measured at 235 nm. One pump of the dual-pump gradient-elution chromatograph contained acetonitrile and the other 0.5% acetic acid in distilled water; an isocratic 42% acetonitrile mixture of the two solvents was used. The flow-rate of the solvent mixture was 70 ml/hr with a column input pressure of 197 atm (2900 p.s.i.). Concentrations between 1.0 and 25 μ g per sample could be measured with a coefficient of variation from 1-6%. 40-50 samples can easily be assayed in a day. The method does not require prior chromatographic preparation or multiple extractions.

Woodhouse et al (56) described a high-performance liquid chromatographic method for measuring plasma concentrations of clofibric acid after administration of clofibrate to humans. 50 μ g of the inter-

nal standard (4-chloro-2-methylphenoxyacetic acid) in methanol and 3M HCl (0.5 ml) were added to plasma (1 ml) in a glass stoppered centrifuge tube, shaken, allowed to stand for 5 min. and then extracted with 6 ml ether. Ether was evaporated to dryness under nitrogen and the residue dissolved in methanol. 10 μ l portions of this solution were injected into the chromatograph using a stop-flow injection technique. The stainless steel column (25 x 0.46 cm i.d.) was packed with C₁₈ Partisil (10 μ m). The mobile phase was 27% acetonitrile containing 0.4% orthophosphate buffer (to maintain the pH at 4.2) at a flow rate of 2 ml/min and a back pressure of 50 bar. Retention times of internal standard and clofibric acid were 6 and 7 min. respectively.

7. Proton Magnetic Resonance Spectrometry:

Hassan and Loutfy (4) have developed PMR analytical method for the quantitation of clofibrate as a drug entity and in capsule dosage form. The four aromatic protons quartet centred at 7.03 ppm (Fig. 3) was chosen for quantitation of clofibrate. Malonic acid was employed as an internal standard, since it exhibits two protons methylene singlet at 3.36 ppm (Fig. 7) which is widely separated from those of clofibrate. Acetone was used as the solvent, since clofibrate and malonic acid are soluble in it and its methyl protons singlet at 2.07 ppm (Fig. 7) does not interfere with the downfield protons of both compounds. The procedure proved to be simple, rapid, accurate and precise. Standard deviations of $\pm 1.07\%$ and $\pm 1.34\%$ were obtained for pure drug and capsules respectively. The PMR spectrum in addition, provides a specific means of identification of clofibrate, and detection of impurities.

Another PMR procedure have been also reported (57) for the determination of clofibrate. For the bulk drug, 100 to 150 mg of sample is shaken with 5 ml of a soln. (10 mg ml⁻¹ of the internal standard (hexamethylcyclotrisilazane) in CCl₄ and the mixture is transferred to a NMR tube. For capsules, the contents are extracted with CCl₄ (4 x 5 ml), the combined extracts are made up to 25 ml with CCl₄ and 5 ml of this soln. is placed in the n.m.r. tube, together with 1 ml of the internal standard soln. (40 to 50 mg ml⁻¹). The NMR spectrum is then

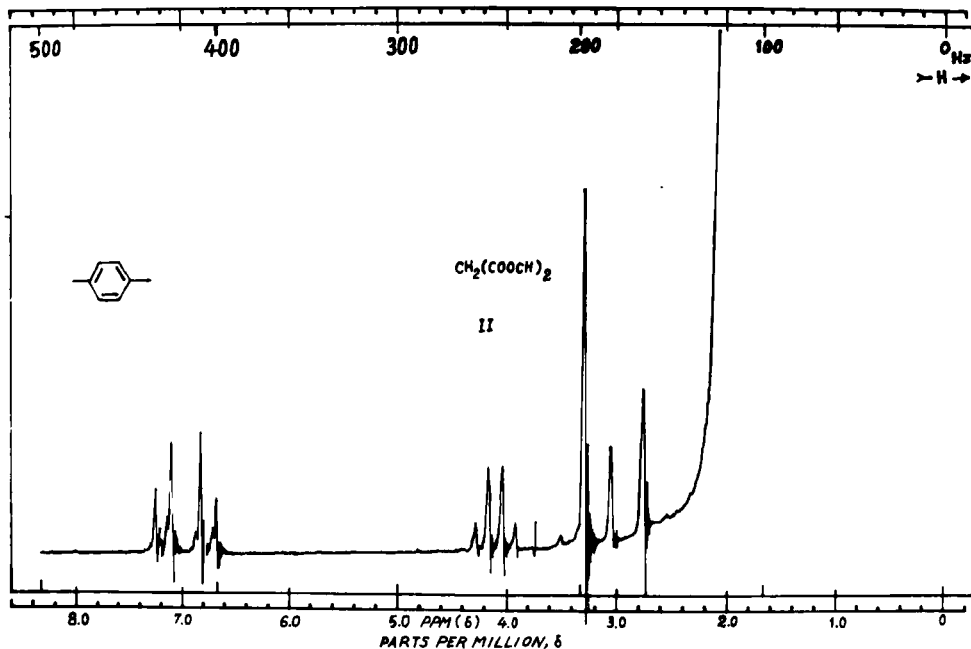


Fig. 7. PMR Spectrum of Clofibrate, Malonic acid and Tetramethylsilane in Acetone.

recorded, and the clofibrate concn. is calculated by comparing the integral of the singlet peak at 1.47 p.p.m. for clofibrate with that for the internal standard. The procedure is simple, rapid and accurate, and the recovery is $99.6 \pm 3.20\%$.

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CLOTRIMAZOLE

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1. Description

1.1 Names, Formula, Molecular Weight and Structure

Clotrimazole was first synthesized in 1969 by Plempel et al. (1) and tested under the name Bay b5097. The compound has been marketed widely under the trade names of Canesten, Lotrimin, Gyne-Lotrimin, and Mycelex.

The chemical name of clotrimazole is 1-(2-chloro-phenyl)diphenylmethyl-1H-imidazole.

The molecular formula of clotrimazole is $C_{22}H_{17}ClN_2$ and the molecular weight is 344.8 g/mol. The structure is given in Figure 1.

1.2 Drug Properties

Clotrimazole is a broad-spectrum antimycotic agent effective against pathogenic dermatophytes, yeasts, and several species of Candida, Trichophyton, Microsporum, Epidermophyton, and Malassezia. Preparations of the drug are used both in the topical treatment of dermal infections and to combat vulvovaginal candidiasis.

Results of initial clinical studies with this compound published in 1969 (2) were followed by more detailed reports outlining the spectrum and mechanisms of clotrimazole activity (1,3,4). At therapeutic concentrations drug action is fungistatic; however, at high concentrations (20 μ g/ml) some in vitro fungicidal activity has been observed (1,4).

1.3 Appearance, Color, Odor and Taste

Clotrimazole is a colorless, odorless, tasteless, crystalline solid.

2. Physical Properties

2.1 Nuclear Magnetic Resonance Spectra

2.1.1 Proton Magnetic Resonance

The proton NMR spectrum (Figure 2) of a 10% (w/v) solution of clotrimazole in deuterated chloroform at ambient temperature was obtained by using a Varian CFT-20 spectrometer operating at a frequency of 79.5 MHz. The chemical shifts given in Table I are downfield from the internal reference tetramethylsilane. Proton assignments are as indicated in Figure 1.

A 220 MHz proton NMR spectrum of a solution of clotrimazole in C_6D_6 has also been reported in the literature (5).

Table I
PMR Spectral Assignments

<u>Protons</u>	<u>Chemical Shifts (δ)</u>	<u>Intensity</u>	<u>Multiplicity</u>
5-H	6.75	1H	triplet, J=1.5 Hz
4-H	7.00	1H	triplet, J=1.5 Hz
2-H	7.40	1H	triplet, J=1.5 Hz
3'-H to 6'-H 2"-H to 6"-H 8"-H to 12"-H	6.95-7.40	14H	broad multiplets
H ₂ O	1.85	--	--

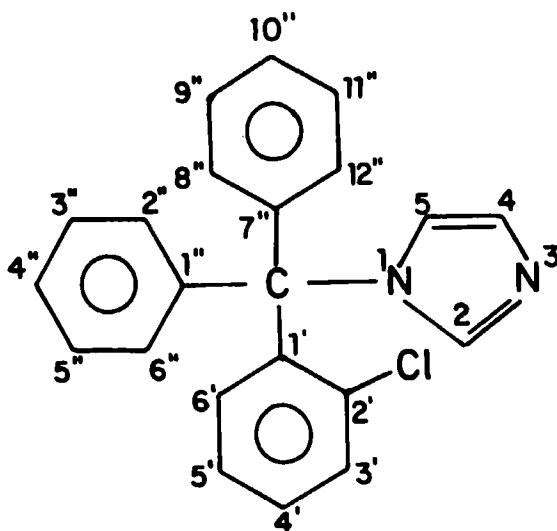
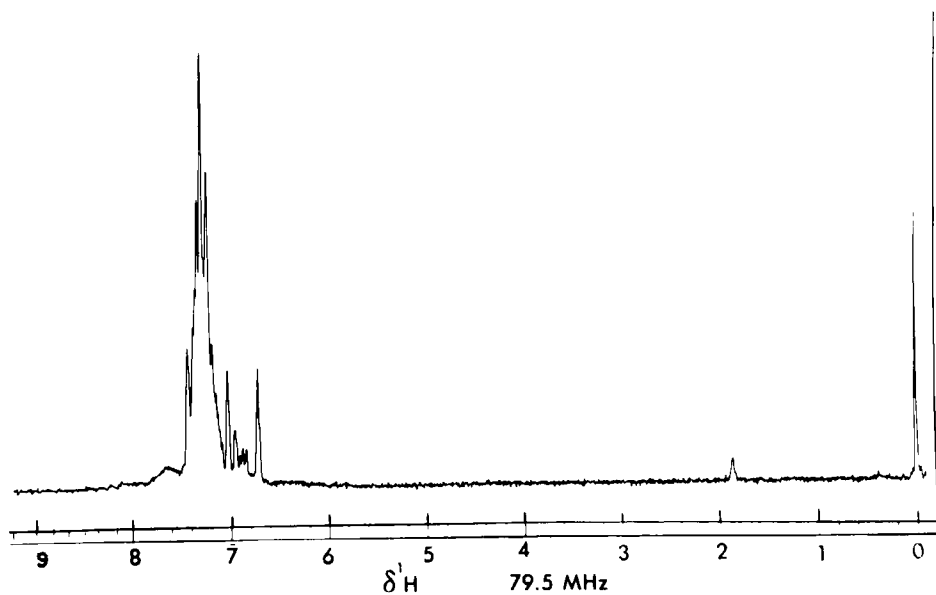


Figure 1. Structure of Clotrimazole.

Figure 2. Proton Nuclear Magnetic Resonance Spectrum of Clotrimazole in CDCl₃.

2.1.2 Carbon-13 Magnetic Resonance

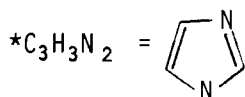
The carbon-13 proton decoupled NMR spectrum (Figure 3) of a 20% (w/v) solution of clotrimazole in deuterated chloroform at ambient temperature was obtained by using a Varian XL-100 spectrometer operating at a frequency of 25.2 MHz. The chemical shifts are in ppm (δ) with reference to internal tetramethylsilane. The carbon-13 NMR spectrum indicates the presence of five quaternary carbons at $\delta = 75.0, 135.6, 139.1, 140.4,$ and 140.9 , and seventeen olefinic carbons at $\delta = 121.5, 127.0, 128.0$ (4 equivalent carbons), 128.1 (3 equivalent carbons), $128.5, 129.8, 130.1$ (4 equivalent carbons), $130.4,$ and 132.2 ppm.

2.2 Mass Spectrum

The mass spectrum (Figure 4) of clotrimazole was obtained by using a Varian MAT CH5 medium resolution single focusing instrument. The electron energy was 70 eV, and the probe and source temperatures used were 140°C and 250°C , respectively. The results are given in Table II.

Table II

<u>m/e</u>	<u>Ions</u>	<u>Losses*</u>
344	M^+	--
309	$(\text{M}-35)^+$	Cl
277	$(\text{M}-67)^+$	$\text{C}_3\text{H}_3\text{N}_2$
242	$(\text{M}-102)^+$	$\text{C}_3\text{H}_3\text{N}_2 + \text{Cl}$
241	$(\text{M}-103)^+$	$\text{C}_3\text{H}_3\text{N}_2 + \text{HCl}$
239	$(\text{M}-105)^+$	$\text{C}_6\text{H}_5 + \text{H}_2\text{CN}$ or $\text{C}_3\text{H}_3\text{N}_2 + \text{HCl} + \text{H}_2$
199	$\text{H}_5\text{C}_6\text{CC}_6\text{H}_3\text{Cl}^+$ and/or $\text{H}_4\text{C}_6\text{CC}_6\text{H}_4\text{Cl}^+$	--
165	$\text{H}_5\text{C}_6\text{CC}_6\text{H}_4^+$	--



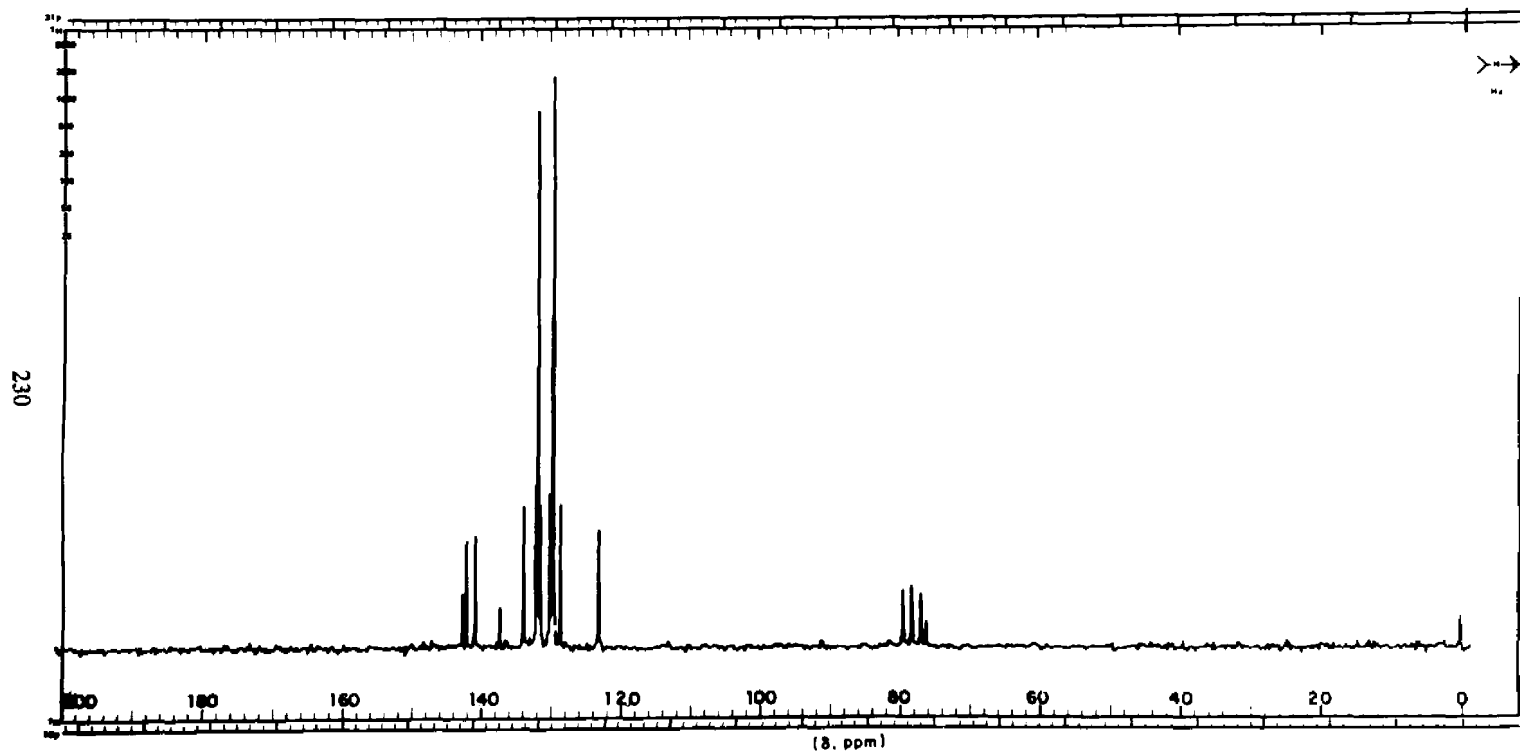


Figure 3. Carbon-13 Nuclear Magnetic Resonance Spectrum of Clotrimazole in CDCl₃.

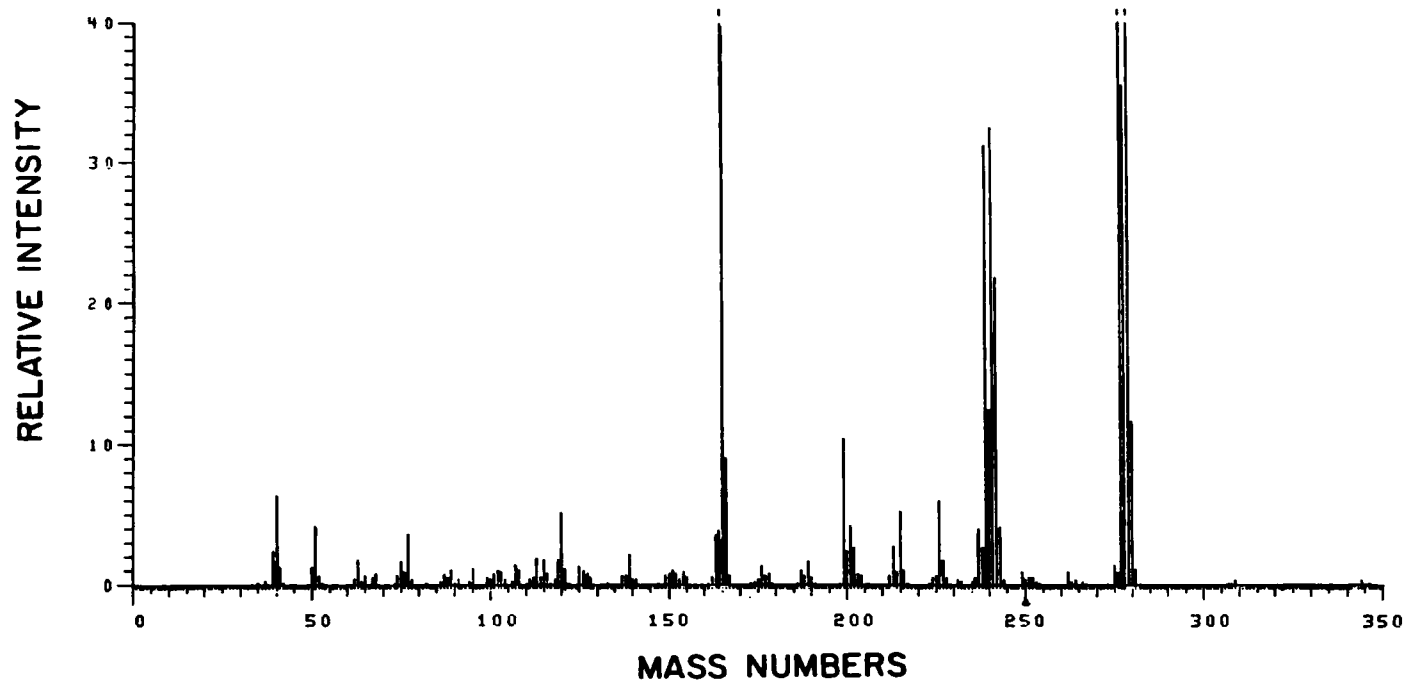


Figure 4. Mass Spectrum of Clotrimazole.

2.3 Infrared Spectrum

The infrared spectrum (Figure 5) of clotrimazole as a dispersion in mineral oil was obtained by using a Perkin Elmer Model 180 infrared spectrophotometer. The major absorption bands are given in Table III.

The infrared spectrum of clotrimazole as a dispersion in potassium bromide has been reported in the literature (5).

Table III
Infrared Band Assignments

<u>Wavenumber (cm⁻¹)</u>	<u>Assignment</u>
3170, 3115, 3085, 3075 (w)	aromatic C-H (stretch)
1585, 1570 (w)	aromatic C=C, C=N (stretch)
1510, 1500, 1450 (m)	aromatic C=C, C=N (stretch)
770, 760, 750 (vs)	aromatic C-H out-of-plane bend
720, 700, 680 (s-vs)	aromatic C-H out-of-plane bend

Notations: vs = very strong; s = strong; m = medium;
w = weak

2.4 Ultraviolet Spectrum

The ultraviolet spectra of clotrimazole in methanol and methanolic 0.1N hydrochloric acid (Figure 6) were obtained by using a Cary Model 118 spectrophotometer. The maxima, minima, shoulders and respective molar absorptivities are given in Table IV.

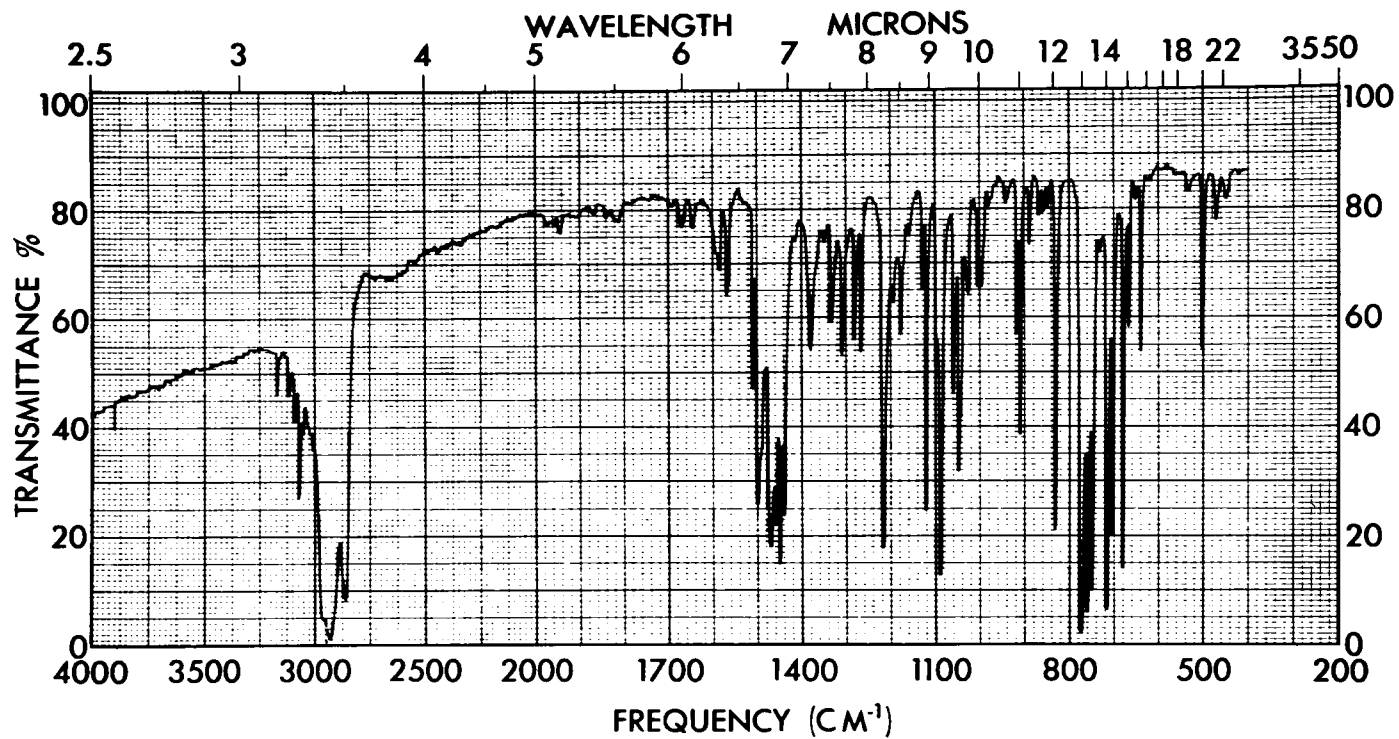


Figure 5. Infrared Spectrum of Clotrimazole in Mineral Oil.

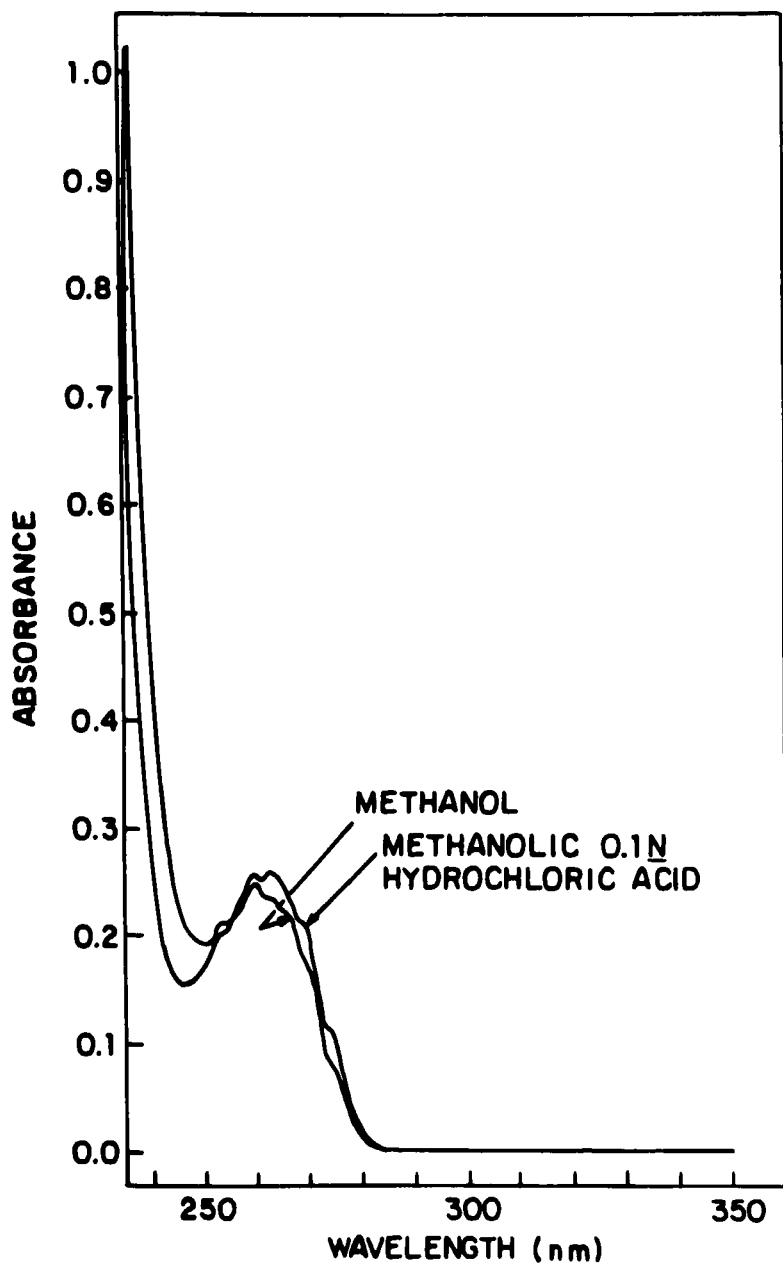


Figure 6. Ultraviolet Spectra of Clotrimazole.

Table IVUltraviolet Spectral Characteristics of Clotrimazole

<u>Solvent</u>	<u>λ (nm)</u>	<u>$\epsilon \times 10^{-2}$</u>
Methanol	274 (shoulder)	1.03
	269 (shoulder)	2.40
	265 (shoulder)	3.32
	263 (shoulder)	3.21
	259 (maximum)	3.40
	253 (maximum)	2.94
	249 (minimum)	2.64
Methanolic 0.1N hydrochloric acid	274 (shoulder)	1.75
	269 (shoulder)	3.28
	263 (maximum)	3.96
	261 (minimum)	3.84
	259 (maximum)	3.93
	254 (shoulder)	3.12
	246 (minimum)	2.38

2.5 Melting Range

The following melting ranges have been reported:

<u>Melting Range</u>	<u>Reference</u>
143 to 144°C	5,6
144 to 145°C	7
141 to 145°C	8

2.6 Thermal Properties2.6.1 Differential Scanning Calorimetry

The differential scanning calorimetry curve (Figure 7) of clotrimazole was obtained by using a DuPont Model 990 Thermal Analyzer at a heating rate of 10°C/minute under a nitrogen atmosphere. A single sharp endotherm was observed with an extrapolated onset temperature of 143°C.

Purity analysis by differential scanning calorimetry (Figure 8) was performed at a heating rate of $1^{\circ}\text{C}/\text{minute}$ under a nitrogen atmosphere. The purity of the sample was determined to be 99.5 mole percent. The latent heat of fusion (ΔH_f) was calculated to be 7540 cal/mol .

2.6.2 Thermogravimetry

Thermogravimetric analysis (Figure 9) of clotrimazole was performed by using a DuPont Model 950 Thermogravimetric Analyzer at a heating rate of $10^{\circ}\text{C}/\text{minute}$ under a nitrogen atmosphere. No weight loss was observed from ambient temperature to about 180°C . The gradual weight loss above 180°C is due to vaporization of the melt.

2.7 Crystal Properties

2.7.1 X-Ray Diffraction

The X-ray powder diffraction pattern (Figure 10) of clotrimazole was obtained by using a Phillips ADP-3500 X-ray Diffractometer and $\text{Cu K}\alpha$ radiation (1.5148 \AA). The data are given in Table V.

2.7.2 Polymorphism

Borka *et al.* (6) have reported the formation of a metastable form of clotrimazole from a crystal film preparation. They report a melting point of 106°C for this metastable form and an infrared spectrum that is different from the stable form.

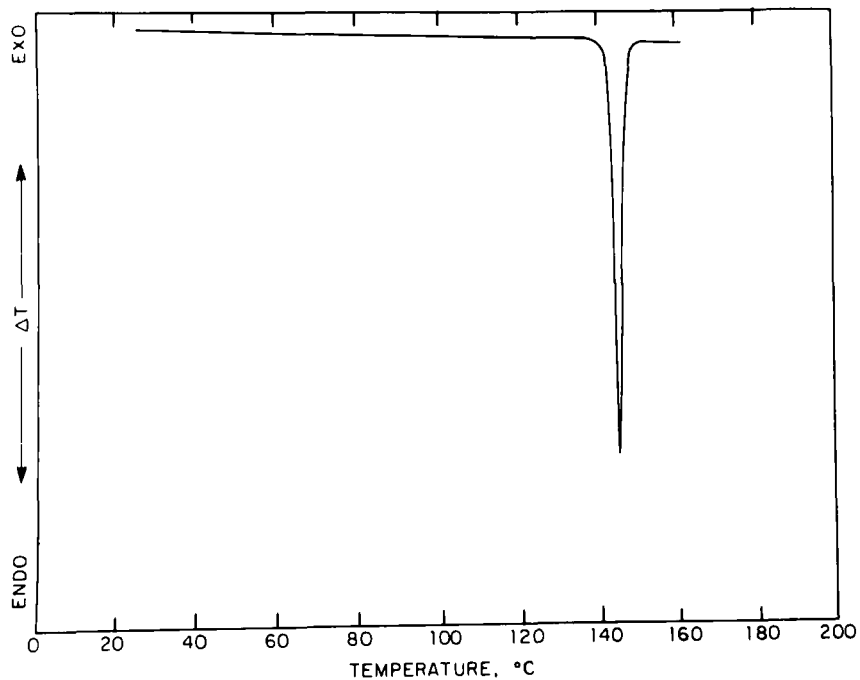


Fig. 7. Differential Scanning Calorimetry Curve of Clotrimazole.

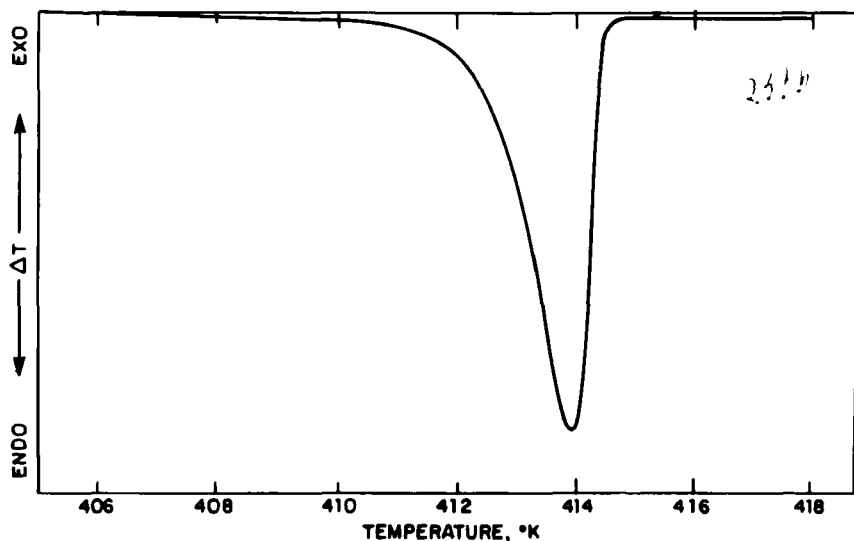


Fig. 8. Differential Scanning Calorimetry Curve of Clotrimazole for Purity Analysis.

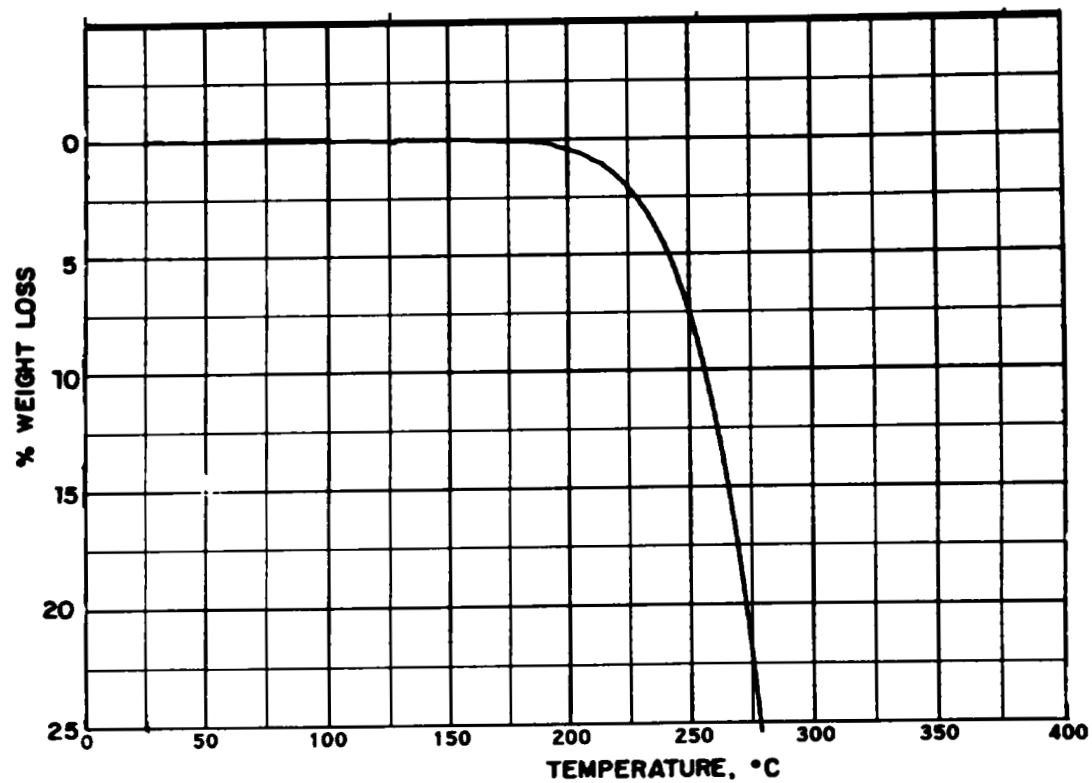


Figure 9. Thermogravimetry Curve of Clotrimazole.

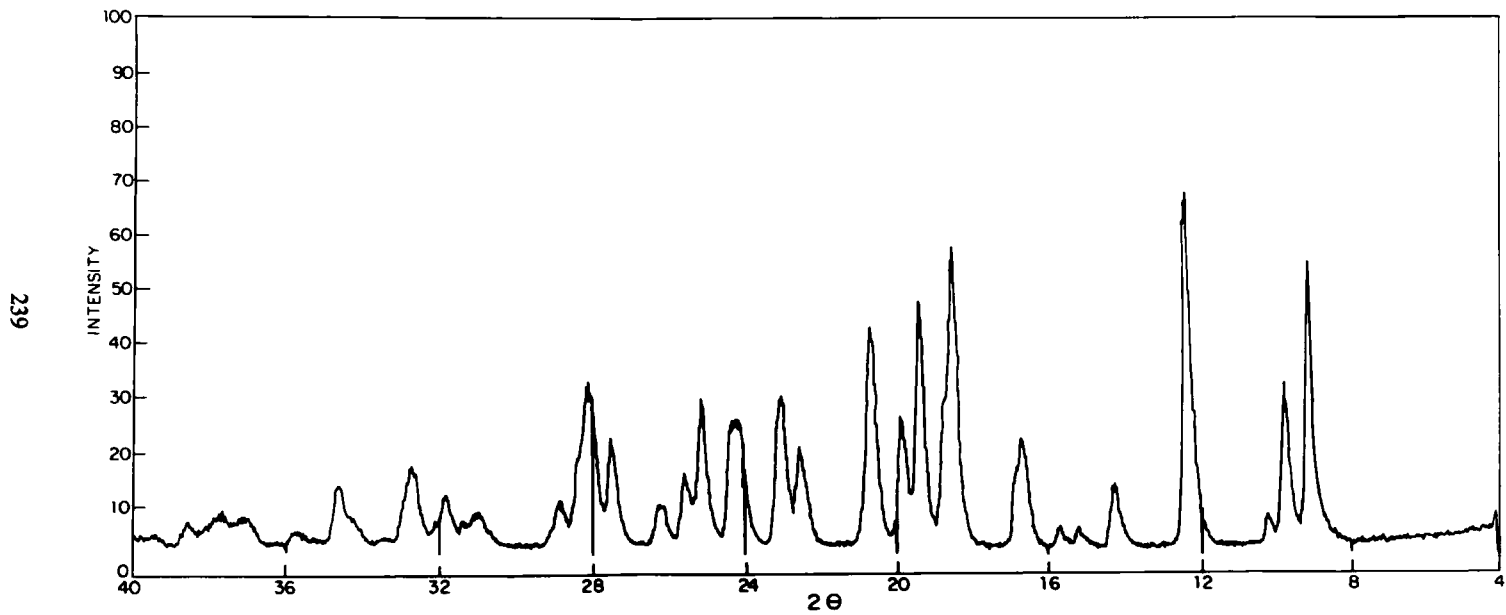


Figure 10. Powder X-ray Diffraction Pattern of Clotrimazole.

Table V
X-Ray Powder Diffraction Pattern of Clotrimazole

2θ	$d(\text{\AA})^a$	I/I'^b
9.172	9.641	80
9.398	9.410	10
9.817	9.009	47
10.203	8.669	10
12.361	7.161	100
14.178	6.247	19
15.159	5.845	7
15.641	5.665	7
16.645	5.326	32
18.517	4.791	82
19.415	4.572	68
19.830	4.477	37
20.647	4.302	61
22.502	3.951	29
22.983	3.870	43
24.101	3.693	36
24.200	3.678	36
24.266	3.668	36
25.086	3.550	41
25.519	3.490	22
26.177	3.404	14
27.474	3.246	32
28.132	3.172	46
28.354	3.148	26
28.810	3.099	14
30.923	2.892	11
30.999	2.885	11
31.318	2.856	9
31.461	2.843	7
31.783	2.815	16
32.655	2.742	23
34.182	2.623	11
34.503	2.599	19
34.577	2.594	19
36.980	2.431	10
37.080	2.424	10
37.196	2.417	9
37.374	2.406	8
37.598	2.392	11
37.680	2.387	11
37.778	2.381	10
37.857	2.376	10
37.989	2.372	8
37.998	2.368	8
38.513	2.337	9

^a d (interplanar distance) = $n \lambda / 2 \sin \theta$

^b I/I' = relative intensity

2.8 Solubility

The solubility of clotrimazole has been determined in solvents by using visual, gravimetric or spectrophotometric analysis (Table VI).

2.9 Dissociation Constant

The pKa of clotrimazole in 50% aqueous ethanol is reported to be 4.7 (5).

Table VI

Solubility of Clotrimazole in Common Solvents

<u>Solvent</u>	<u>Measured Solubility (mg/ml at 25°C)</u>	<u>Method</u>
Acetone	50	gravimetric
Benzene	>100	visual
Chloroform	>100	visual
Diethyl ether	14	gravimetric
Dimethylformamide	>100	visual
Dimethylsulfoxide	45	spectrophotometric
Ethanol USP	95	gravimetric
Ethyl Acetate	45	gravimetric
Methanol	>100	visual
Mineral oil	0.8	spectrophotometric
Petroleum ether	1.1	spectrophotometric
Polyethylene glycol 400	60	spectrophotometric
Propylene glycol	35	spectrophotometric
Water	<0.01	spectrophotometric

3. Synthesis

Clotrimazole is synthesized by the reaction of o-chlorotriptylchloride with imidazole in the presence of a tertiary amine, as described by Buechel, et al. (5) as shown in Figure 11. The yield in this synthesis is solvent-dependent; reactions in solvents with high dielectric constants give the higher yields.

Maul and Scherling (9) used barium $\{^{14}\text{C}\}$ carbonate as starting material to synthesize ^{14}C -clotrimazole. They made the intermediates 2-chloro- $\{\text{carboxyl-}^{14}\text{C}\}$ benzoic acid, 2-chloro- $\{\text{carboxyl-}^{14}\text{C}\}$ benzoylchloride, 2-chloro- $\{\text{carboxyl-}^{14}\text{C}\}$ benzophenone, (2-chlorophenyl)diphenyl - $\{^{14}\text{C}\}$ methanol and (2-chlorophenyl)diphenyl - $\{^{14}\text{C}\}$ -methylchloride en route to radiolabelled clotrimazole, 1-(2-chlorophenyl)diphenyl- $\{^{14}\text{C}\}$ - methyl-1H-imidazole.

4. Stability

Clotrimazole is stable in the solid state under normal storage conditions. It is unaffected by heat (70°C) and exposure to daylight for up to two weeks (10).

In solution, the stability of clotrimazole is pH dependent. In an alkaline medium it is stable, but hydrolyzes in an acidic medium to (o-chlorophenyl)-diphenylmethanol plus imidazole. Buechel et al. report on the relative hydrolytic stability of Clotrimazole in solution in ethanol-water and isopropanol-water mixtures under acidic, neutral, and alkaline conditions (4).

Thermal and light pH-stability studies have been performed. Known amounts of clotrimazole were sealed in glass ampuls after the addition of 10 ml of an aqueous buffer. The pH range studied was 1 to 13. Table VII gives the results for samples stored at 75° , 85° , and 95°C for one week. Results are given in Table VIII for samples stored in the dark and under 350 foot-candles of fluorescent light for three months. Analyses were performed by using thin-layer chromatography and elution followed by UV spectral analysis (10).

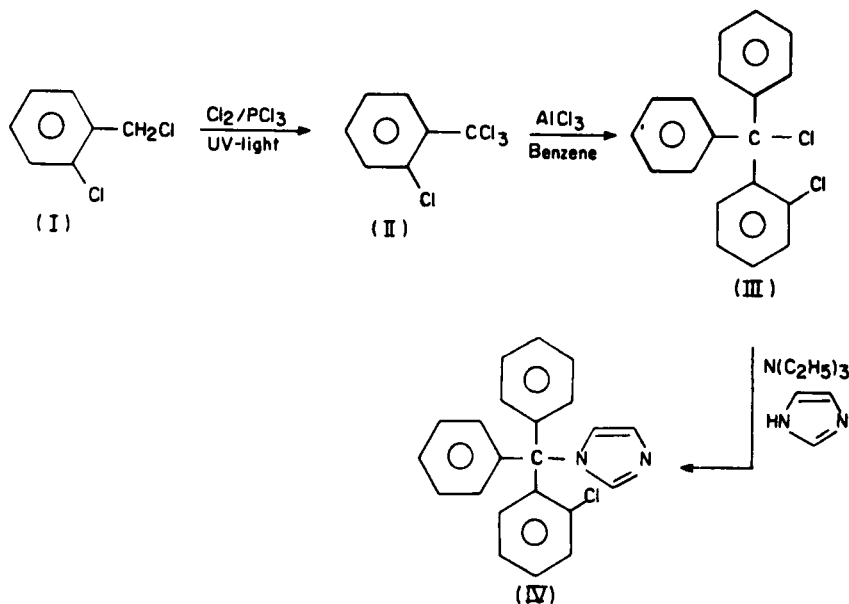


Figure 11. Synthetic Pathway to Clotrimazole

- I. 2-chlorobenzylchloride
- II. 2-chlorobenzotrichloride
- III. 2-chlorotritylchloride
- IV. Clotrimazole

Table VII

pH-Thermal Stability Profile

pH	Buffer	State	Recovery of Clotrimazole in Percent*		
			75°C	85°C	95°C
1	Hydrochloric acid	solution	0	0	0
2	Citrate	part solution	0	0	0
4	Citrate	suspension	84	55	4
4	Acetate	suspension	84	65	6
6	Phosphate	suspension	101	98	90
7	Phosphate	suspension	105	101	94
8	Phosphate	suspension	105	101	105
10	Borate	suspension	102	102	100
13	Sodium hydroxide	suspension	101	105	102

*After one week.

5. Drug Metabolism and Pharmacokinetics

5.1 Drug Metabolism

After oral or topical administration clotrimazole undergoes rapid biotransformation into inactive metabolites. Duhm and co-workers (11) isolated five clotrimazole metabolites from rat urine and bile but found no clotrimazole. Structures of the metabolites are shown in Figure 12.

Human urine and serum tested after oral doses of clotrimazole contain only trace amounts of the drug substance. The two major metabolites found in urine, serum, and bile are (2-chlorophenyl)(4-hydroxyphenyl)-phenylmethane and (2-chlorophenyl)bis-phenylmethane; a smaller amount of (2-chlorophenyl)bis-phenylmethanol is also present.

5.2 Pharmacokinetics

Early clinical studies employing oral administration of clotrimazole provided data on the pharmacokinetics of systemically distributed drug substance in humans. As discussed above, metabolism of clotrimazole is extremely rapid, and reliable pharmacokinetics data have been obtained only by using analytical methods which determine both the drug substance and metabolites. In experiments with ¹⁴C-labelled drug, Duhm *et al.* (11) found that no clotrimazole was detected in serum for a 20 minute interval after dosing. Peak serum levels of up to 4 µg/ml were confirmed by a number of workers (1,12-15); these levels were reached between two and four hours after dosing in adults and at about six hours after administration to children. Rosenkrantz and Puetter (16) estimated that up to 98% of clotrimazole in serum is bound to serum proteins.

Several workers have followed systemic clotrimazole clearance by determining the drug and metabolites excreted in urine. Weingaertner, *et al.* (17) found no drug substance in urine until between 30 and 60 minutes after administration; they reported peak urine concentrations at between 6 and 12 hours. Plempel and co-workers (1) have reported peak urine concentrations of 30 to 60 µg/ml 12 to 14 hours after dosing.

Table VIII
pH-Light Stability Profile

pH	Buffer	State	Recovery of Clotrimazole in Percent**	
			Light	Dark
1	Hydrochloric acid	solution	9	12
2	Citrate	part solution	20	41
4	Citrate	suspension	98	102
6	Phosphate	suspension	97	103
7	Phosphate	suspension	99	102
8	Phosphate	suspension	101	103
10	Borate	suspension	99	103
13	Sodium hydroxide	suspension	103	99

**Stored in the dark and under 350 foot-candles of fluorescent light for 3 months.

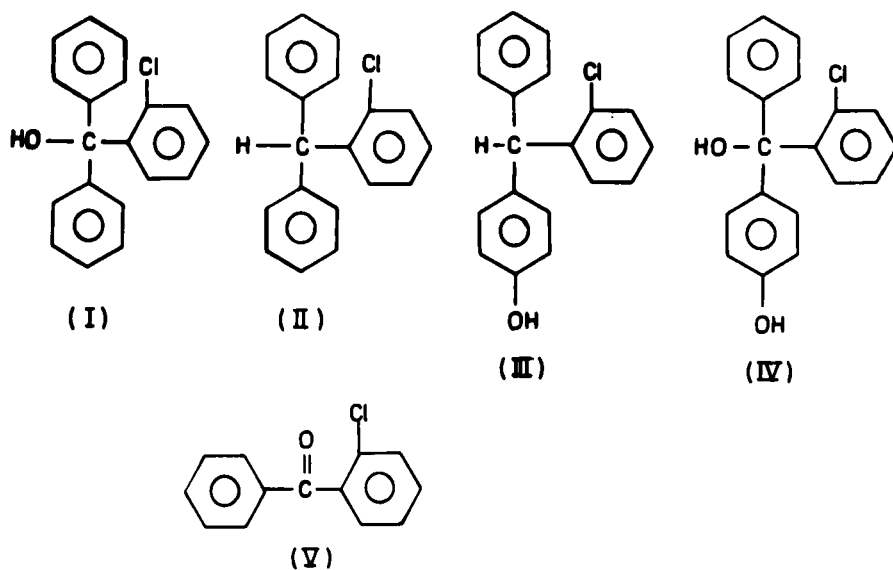


Figure 12. Clotrimazole Metabolites:

- I. (2-chlorophenyl)diphenylmethanol
- II. (2-chlorophenyl)diphenylmethane
- III. (2-chlorophenyl), (4-hydroxyphenyl)phenylmethane
- IV. (2-chlorophenyl), (4-hydroxyphenyl)phenylmethanol
- V. 2-chlorobenzophenone

Pharmacokinetics of topically applied clotrimazole has been studied in several laboratories. Duhm and co-workers (18) found that while ^{14}C -clotrimazole applied as a cream penetrated the skin to a depth of 2000 μm , no drug substance or metabolites were detected in the serum. A small quantity of the material (up to 0.4%) was detected in the urine over a five-day period. In a similar study, Holt (19) used a microbiological assay with a detection limit of 0.01 $\mu\text{g/ml}$; over a thirty-day period of clotrimazole cream application he observed no drug substance in either serum or urine. Wallace, et al. (20) noted that 24 hours after a ten-day treatment with clotrimazole topical preparations, drug levels in the skin were as high as 2 $\mu\text{g/mg}$; this value decreased to about 0.4 $\mu\text{g/mg}$ after four days.

In studies using clotrimazole vaginal tablets, 100 mg, Duhm et al. (18) found peak serum levels of 0.03 $\mu\text{g/ml}$ after 24 hours.

Pharmacokinetics and pharmacology of clotrimazole are further covered in a comprehensive summary by Sawyer, et al. (21) as well as in reviews by Meade (22) and Seneca (23).

6. Methods of Analysis

6.1 Elemental Analysis

Conventional procedures for the determination of C, H, N, and Cl yielded the following results for a sample conforming to USP XX specifications.

<u>Element</u>	<u>Found</u>	<u>%</u>	<u>Theory</u>
C	76.63		76.63
H	4.93		4.97
N	8.12		8.12
Cl	10.18		10.28

6.2 Identification

Several methods have been proposed for the identification of clotrimazole. Kuhnert-Brandstaetter, et al. (8) observed that the drug substance forms eutectics with phenacetin and benzanilide which melt at 110° and 115°C, respectively. Data on eutectic melting points is used to confirm the identity of clotrimazole, which melts at 143°C.

Kráčmar, et al. (24) have characterized the ultraviolet spectra of clotrimazole in methanol and in 0.1N HCl as an aid to the identification of this drug substance.

The USP XX identification tests for clotrimazole (25) include both thin-layer chromatography, in which the sample spot must appear at the same R_f value as reference standard material, and infrared spectrophotometry.

6.3 Spectrophotometric Analysis

Limited use has been made of ultraviolet spectrophotometry in the analysis of clotrimazole due to its low absorptivity. Kráčmar, et al. (24) suggested that such analyses should be possible; Szabolcs (26) used measurements at 261 nm to assay clotrimazole in formulations.

Upon heating with trichloroacetic or perchloric acids, solutions of clotrimazole become bright yellow; the color fades rapidly with trichloroacetic acid but persists with perchloric acid (1). This reaction, which forms the basis for a colorimetric assay of the drug substance, gives solutions which obey Beer's law up to concentrations of 10 µg/ml when read at 436 nm (27). This method has been used for the estimation of clotrimazole in biological fluids and tissues after either solvent extraction (16,27) or extraction followed by thin-layer chromatography (1,13,17,28).

6.4 Titrimetric Analysis

Assay of clotrimazole bulk drug substance is performed by nonaqueous titration (25). The sample dissolved in glacial acetic acid is titrated with 0.1M perchloric acid in glacial acetic acid to a green end-point; p-naphtholbenzein is used as indicator. Each milliliter of 0.1M perchloric acid is equivalent to 34.48 milligrams of clotrimazole.

After extraction with chloroform, Szabolcs (26) assayed clotrimazole in pharmaceutical formulations by titration with 0.1M perchloric acid; gentian violet was used as indicator.

A two-phase titration method developed by Pellerin, et al. (29-31) serves as a basis for the titration of clotrimazole with sodium lauryl sulfate. Samples of clotrimazole formulations are partitioned between chloroform and 2N sulfuric acid. Methyl yellow is added as indicator and the mixture titrated with standardized sodium lauryl sulfate in water. The end point is reached when the chloroform layer turns gold-orange (32). This stability-indicating titration has been used to assay clotrimazole in formulations (32,33) and to follow hydrolysis of the drug substance (5).

6.5 Chromatographic Analysis

6.5.1 Paper Chromatography

Clotrimazole can be separated from its impurities and degradation products by descending paper chromatography (10). The method uses paper impregnated with propylene glycol and a mobile phase of propylene glycol-saturated ligroin. Clotrimazole is detected at $R_f = 0.4$ by spraying with Dragendorff reagent.

6.5.2 Thin-Layer Chromatography

Thin-layer chromatography (TLC) on silica gel has been used extensively to separate clotrimazole from formulation and biological matrices prior to quantitation. A summary of thin-layer adsorbents and mobile phases used in these separations is given in Table IX.

Table IX
Thin-Layer Chromatography Systems for Clotrimazole

<u>Plate Medium</u> (see below)	<u>Solvent</u> (see below)	<u>Detection</u> (see below)	<u>Rf Value</u>	<u>Reference</u>
a	1st Dimension: 2nd Dimension:	A B I	0.57 0.64	9
b	1st Development: 2nd Development:	C D II	-	17
b	1st Development: 2nd Development:	C E II	-	13
b	1st Development: 2nd Development:	C D III	0.4	27
b	F	III	0.55	27
b	C	II	0.3	1,28
c	G	IV	0.65	25
c	H	IV,V	0.4	25
c	J	IV,V	0.85	10

Table IX (continued)Plate Medium

- a. Silica gel 60.
- b. Silica gel foil (Polygram Sil NH.R, Machery-Nagel).
- c. Silica gel 60 F.

Solvent System

- A. Benzene:methanol (4:1).
- B. Methanol.
- C. Chloroform.
- D. Petroleum ether (40-60⁰):acetone:
benzene:ethanol:pyridine
(70:12:10:7:1).
- E. Benzene.
- F. Petroleum ether (40-60⁰):ethyl-
acetate:acetone:ethanol:
ammonia (25%) (45:25:25:5:0.5).
- G. Xylene:n-propanol:ammonia (180:20:1).
- H. Ether equilibrated with ammonia vapor.
- J. Ethyl acetate:ammonia (25%) (98:2).

Detection Method

- I. Autoradiography.
- II. Trichloroacetic acid in n-butylacetate.
- III. Spray sequentially with ethanolic iodine, sodium carbonate, and sulfanilic acid/sodium nitrite.
- IV. Fluorescence quenching under short-wave ultraviolet light.
- V. Spray with Dragendorff reagent.

Quantitative analyses employing TLC have been carried out in several ways. Ritter, *et al.* (27) used densitometry to determine clotrimazole on the TLC plate after formation of a red sulfanilic acid derivative. Their calibration curves were linear between 0.5 and 4 μg clotrimazole per spot. Other investigators have determined the drug substance spectrophotometrically by scraping the bands off the TLC plate and reacting either with butyl acetate and perchloric acid (1,13,17,28) or with bromphenol blue (10).

6.5.3 Gas Chromatography

Clotrimazole in human skin samples has been determined by gas chromatography with electron-capture detection (20,34). Samples were extracted with ether, dried, redissolved in benzene and chromatographed on 6', 1/8" glass columns packed with 3% OV-17 on Gas Chrom Q. Column temperature was 250°C and the carrier gas was argon-methane at 9 ml/min. Standard curves were linear over the range of 1-25 ng injected.

6.5.4 High Performance Liquid Chromatography

Stability-indicating assays of clotrimazole in the bulk drug substance and in formulations have been carried out by high performance liquid chromatography (35). The analyses were performed on a μ Bondapak C18 column with a mobile phase of methanol:0.025M K_2HPO_4 (3:1) at 1.0 ml/min. Chromatographic response was linear from 2 to 40 μg clotrimazole injected. Average recovery of drug substance from formulated materials ranged from 99.5 to 100.0 percent. Analyses were reproducible, with between-day relative standard deviations between 0.6 and 1.8 percent.

6.6 Radiochemical Analysis

Duhm, *et al.* (18,36) used ^{14}C -labelled clotrimazole to determine drug distributions in biological samples. Radioactivity was determined in serum and urine by the use of liquid scintillators containing 8 g/l of butyl PBD in toluene:dioxane:ethanol (1:1:1). Skin samples were burned; the resulting $^{14}\text{CO}_2$ was absorbed in base and counted by means of scintillators.

6.7 Microcalorimetric Analysis

Inhibition of the respiration of Saccharomyces cerevisiae by antifungal agents provides the basis for a sensitive clotrimazole assay. Beezer and co-workers (37) monitored respiration microcalorimetrically before and after addition of clotrimazole; degree of inhibition of respiration was related to the concentration of clotrimazole. The minimum drug concentration detectable by this method was 3×10^{-5} M.

6.8 Microbiological Analysis

Microbiological assays of clotrimazole are agar diffusion assays based on a comparison between the growth inhibition zones produced by standard solutions and those produced by test samples. The test organism Candida pseudotropicalis var. carshalton has been used by several investigators (12,27,38) to assay clotrimazole in serum, urine, and feces. When testing the stability of clotrimazole in culture medium, Hoeprich and Huston (39) employed Kluveromyces fragilis as test organism. In a study of clotrimazole stability on sample discs, Saubolle and Hoeprich (40) used Candida albicans as test organism. Holt (41) has discussed the use of several organisms and assay types in the analysis of antifungal drugs, including clotrimazole.

Two papers have compared microbiological assay results to thin-layer chromatographic determinations of clotrimazole (27,42).

7. Acknowledgements

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Literature survey terminated December, 1980.

DOPAMINE HYDROCHLORIDE

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and David M. Baaske*

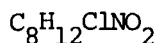
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1. Description

1.1 Chemical and Proprietary Names

Dopamine hydrochloride is the non-proprietary name for 4-(2-aminoethyl)-1,2-benzenediol hydrochloride. The free base (dopamine) has also been known in the chemical literature as 3-hydroxytyramine and 3,4-dihydroxyphenethylamine. The drug is available generically for the correction of hemodynamic imbalances present in the shock syndrome due to myocardial infarction, trauma, endotoxic septicemia, open heart surgery, renal failure and chronic cardiac decompensation as in congestive heart failure (1). Proprietary names include Cardiosteril, Docard, Dopamine Fabre, Dopamine Gullini, Dopastat, Dynatra, Intropin, Intropinect, Intropine and Orion.

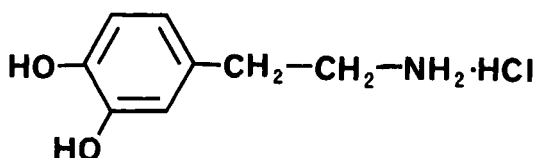
1.2 Empirical Formula



Molecular Weight

189.64

Structure



1.3 Appearance, Color & Odor

Dopamine hydrochloride is a white to off white odorless crystalline powder.

2. Physical Properties

2.1 Melting range

The Merck Index reports the melting point of the hydrochloride salt as 241°C with decomposition (2). The

equilibrium melting range (in the absence of air) has been established as 245°C - 246°C with decomposition (3).

2.2 Solubility Profile

Dopamine hydrochloride is freely soluble in water; soluble in methanol and hot ethanol; practically insoluble in ether, chloroform, benzene and toluene. Dopamine hydrochloride is soluble in aqueous solutions of alkali hydroxides.

2.3 Infrared Spectrum

The KBr pellet infrared spectrum of 0.5% dopamine hydrochloride obtained with a Perkin-Elmer 283 Infrared Spectrophotometer is contained in Figure 1. The broad strong absorption bands appearing at 3350 cm^{-1} and 3230 cm^{-1} are due to the O-H and N-H stretching vibrations. The intermolecular and intramolecular hydrogen bonding shifts these absorption bands to lower frequencies than a free hydroxyl group ($3700 - 3500\text{ cm}^{-1}$) and a free amine ($3500 - 3300\text{ cm}^{-1}$). The aromatic ($3100 - 3000\text{ cm}^{-1}$) and aliphatic ($3000 - 2900\text{ cm}^{-1}$) C-H stretching bands are present as expected. The N-H bending vibrations of the NH_2 group show medium absorption centered at 1610 cm^{-1} . The C-H out of plane bending of the 1,2,4 trisubstituted benzene ring is indicated by the sharp bands at 876 cm^{-1} and 812 cm^{-1} .

2.4 Ultraviolet Spectrum

Dopamine hydrochloride exhibits a single absorption peak centered at 280 nm with a molar absorptivity (ϵ) of 2707 (Figure 2). The spectrum was obtained with a Beckman Acta III double beam spectrophotometer.

2.5 Proton Magnetic Resonance Spectrum

The 60 MHz proton magnetic resonance spectrum was obtained with a Varian Associates T-60A spectrometer. The spectrum in CD_3OD with tetramethylsilane (TMS) as internal reference is contained in Figure 3. The splitting patterns are not simple. However, the integration and general locations of peaks are consistent with the structure. The peaks at 0.8 and 3.35 are due to small amounts of CH_3OH in the deuterated solvent.

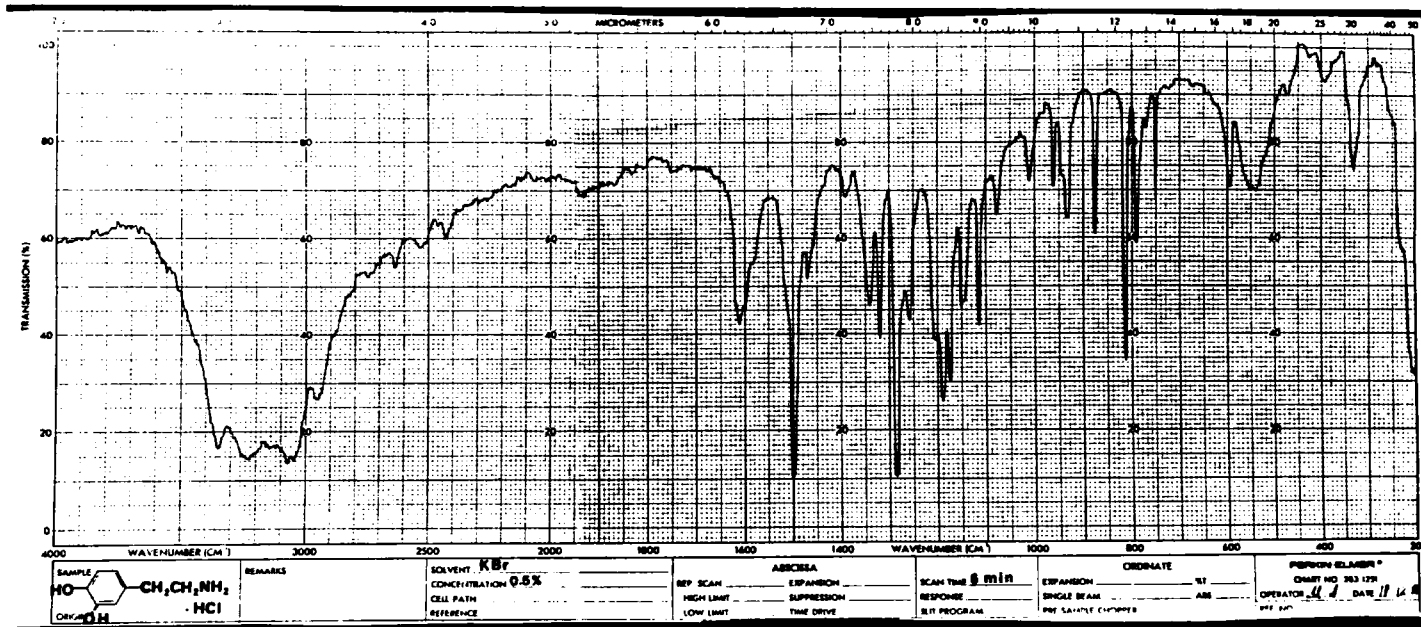


Fig. 1 KBr Infrared Spectrum of Dopamine Hydrochloride.
Instrument: Perkin-Elmer Model 283

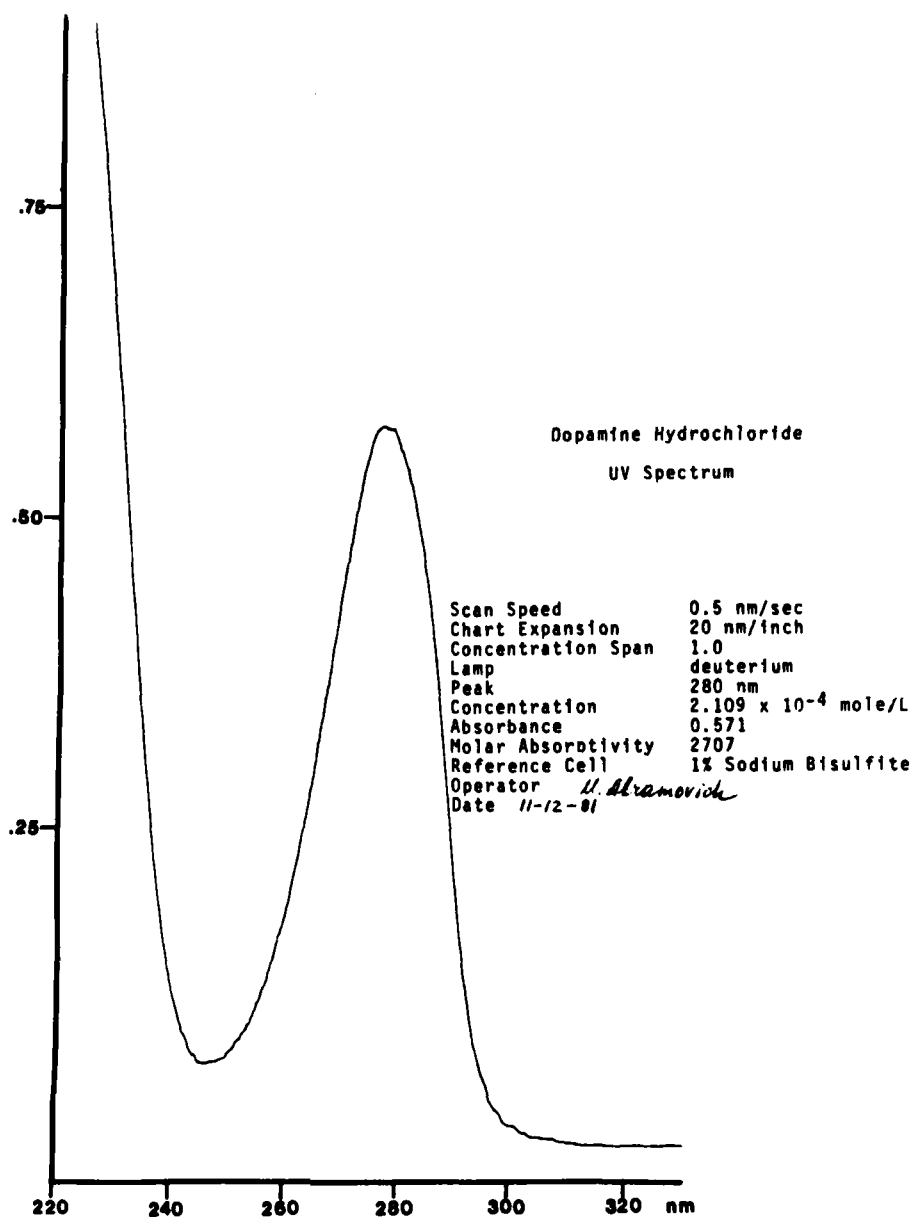


Fig. 2 Ultraviolet spectrum of Dopamine Hydrochloride
Instrument: Beckman Acta III

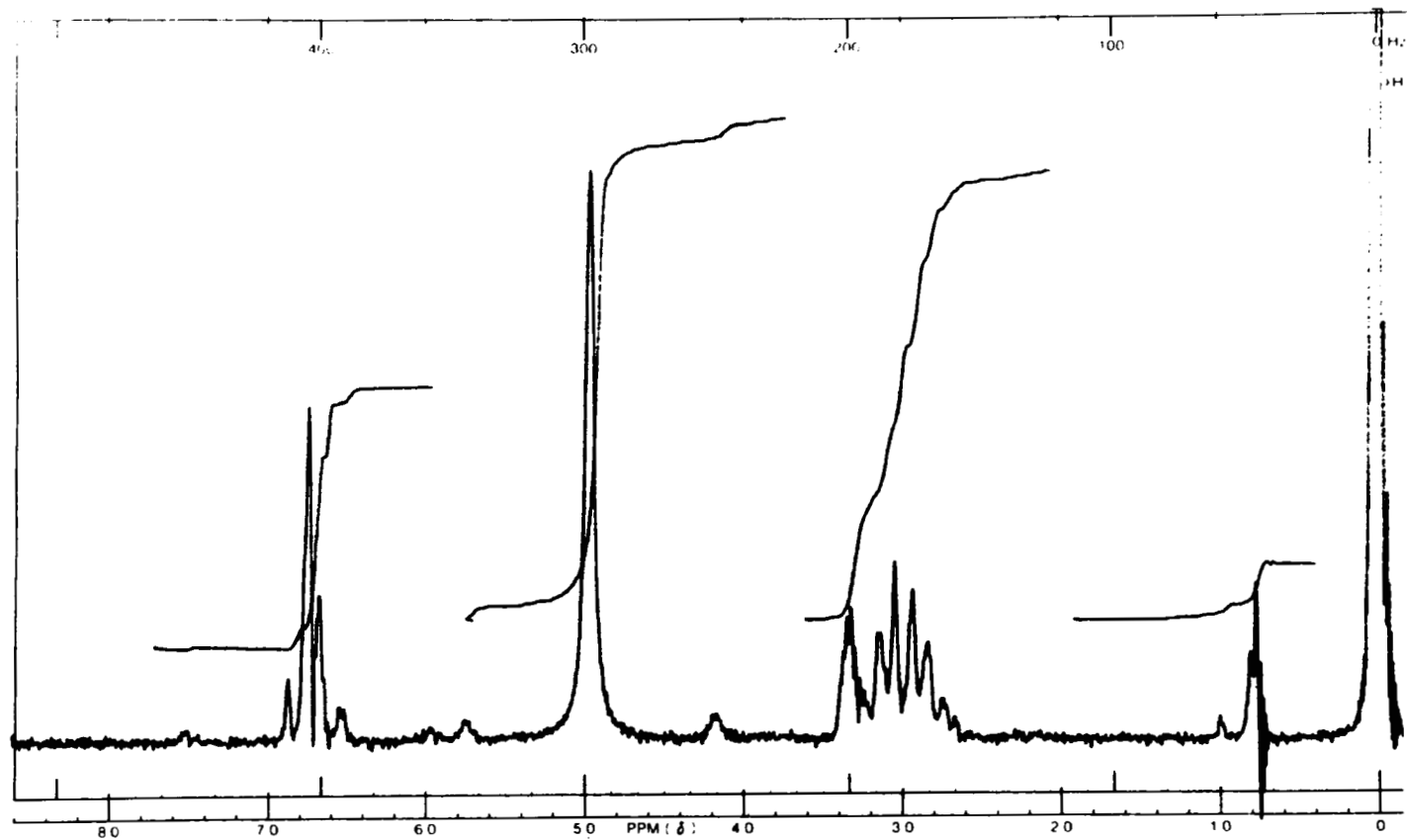
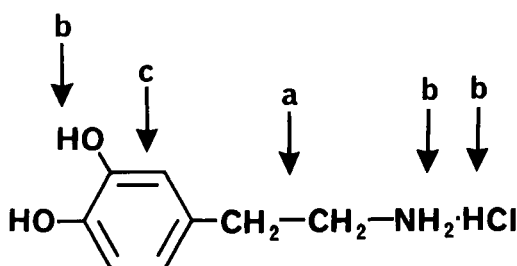


Fig. 3 Proton Magnetic Resonance Spectrum of Dopamine Hydrochloride in CD₃OD
Instrument: Varian T-60A

Chemical shifts (δ) in ppm relative to TMS are:



<u>Proton Assignment</u>	<u># of Protons</u>	<u>Chemical Shift (δ)</u>	<u>Multiplicity</u>
a	4	3.0	multiplet
b	5	4.95	multiplet
c	3	6.75	multiplet

2.6 ^{13}C Magnetic Resonance Spectrum

The ^{13}C analysis of dopamine hydrochloride was taken on a JEOL FX-270 superconducting NMR operating at a frequency of 67.83 MHz using a 45° (9 usec) pulse, a 3.6 second repetition rate and 16384 data points (4).

The sample was dissolved in D_2O ; p-dioxane was added as a reference. Spectra of the entire ^{13}C range were obtained with complete decoupling and gated decoupling with Nuclear Overhauser Effect (NOE) to obtain the chemical shifts and coupling constants. The completely decoupled spectrum is shown in Figure 4.

The resolution of all aromatic carbons (not shown in Figure 4) was obtained using a 2.5 kHz width. The assignment of the aromatic carbon lines was aided by comparison to 3,4 dihydroxybenzene.

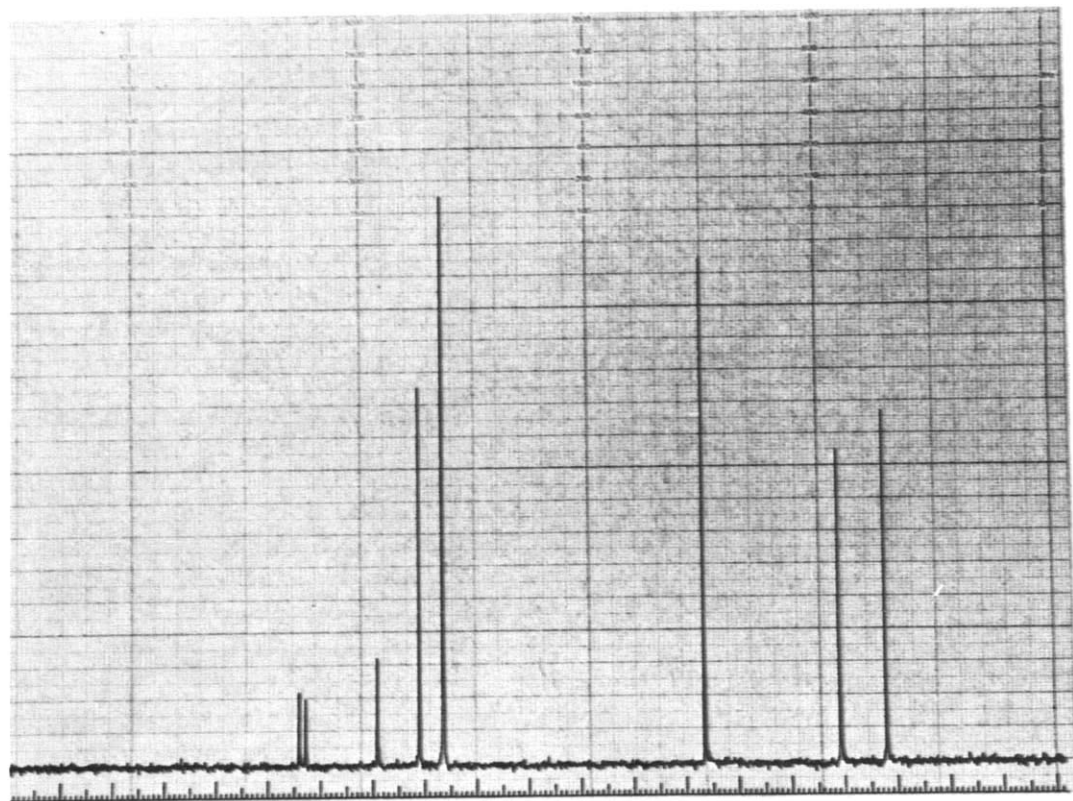


Fig. 4 ^{13}C Magnetic Resonance Spectrum of Dopamine Hydrochloride with p-dioxane as reference.
Instrument: JEOL FX 270

With the carbons numbered as indicated below the chemical shifts and coupling constants are as shown in Table I.

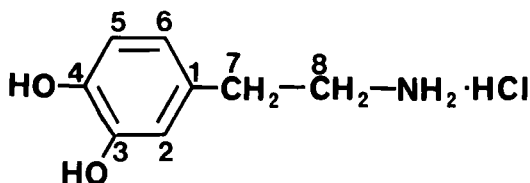


Table I. ^{13}C assignments and C-H coupling constants for Dopamine Hydrochloride

Carbon Number	Shift (ppm)	J_{CH} (Hz)
1	129.1	-
2	116.3	159.3
3	143.9	-
4	142.7	-
5	116.4	156.7
6	121.1	160.0
7	32.0	128.3
8	40.7	143.9

2.7 Mass Spectra

The electron impact (EI) mass spectrum at 70 eV and the methane derived chemical ionization (CI) mass spectrum were obtained with a Hewlett-Packard 5985 quadrupole mass spectrometer (5).

The CI spectrum contains two prominent ions at m/e 137.0 and m/e 154.0. The m/e 154.0 corresponds to $\text{C}_8\text{H}_{12}\text{NO}_2^+$ which is protonated dopamine. Loss of ammonia leaves $\text{C}_8\text{H}_9\text{O}_2^+$ which is the base peak in the spectrum at m/e 137.0.

The EI spectrum is surprisingly simple. The base peak at m/e 124.1 corresponds to $C_8H_8O_2^+$. The intact dopamine at m/e 153.0 is seen in the spectrum. The m/e 77.0 and m/e 78.1 are due to the aromatic ring and correspond to $C_6H_5^+$ and $C_6H_6^+$ respectively. The hydrochloride salt is not seen in either the EI or CI spectrum which is as expected.

2.8 Differential Scanning Calorimetry

Dopamine hydrochloride was heated at a rate of 10° per minute in a Perkin-Elmer DSC-2 differential scanning calorimeter from $460^\circ K$ to $530^\circ K$. A single sharp endotherm was observed with an onset temperature of $516^\circ K$ ($243^\circ C$) and with the endotherm maximum at $519.8^\circ K$ ($246.8^\circ C$). An accurate heat of transition (ΔH) could not be calculated because of decomposition upon melting.

2.9 Crystal Properties

Crystals from a representative lot of dopamine hydrochloride are predominantly triangular plates (3). The optical crystallographic examination indicated that the crystals are triclinic or possibly monoclinic.

X-ray powder patterns were obtained with copper radiation and a nickel filter. The d -spacings and relative intensities (I/I_0) are listed in Table II. The first column shows the pattern for the drug after grinding to break up the plates and therefore the preferred orientation of the crystals. Column 2 shows the diffraction pattern of the crystals without prior grinding. The absence of several key diffraction lines and the reduced influence of others in this orientation is clearly demonstrated by comparing the two columns.

TABLE II

X-ray Diffraction Powder Patterns of Dopamine Hydrochloride

Ground Sample		Unground Sample	
d-spacing	I/I ₀	d-spacing	I/I ₀
<hr/>			
6.37	5		
5.56	30	5.56	10
5.50	60		
5.24	5	5.24	20
4.57	75	4.58	15
4.18	95	4.19	30
4.07	30	4.07	15
3.98	100	3.97	15
3.81	65	3.82	100
3.72	60	3.71	15
3.53	70	3.53	30
3.49	45	3.49	65
3.43	70	3.43	20
3.23	65	3.23	20
3.19	45		
2.96	10	2.97	5
2.83	10	2.83	5
2.77	55	2.77	15
2.75	45		
2.69	10		
2.63	60		
2.62	60	2.62	80
2.55	60		
2.41	10	2.55	15
2.27	10		
2.17	20	2.17	10
1.90	10	2.09	5
		1.99	5
1.86	10	1.96	5
		1.92	5
		1.90	5

3. Synthesis

Dopamine hydrochloride is available from a variety of commercial sources. Details of the synthetic process are considered proprietary information. However, one workable process using known reactions and readily available materials is shown in Figure 5 (6).

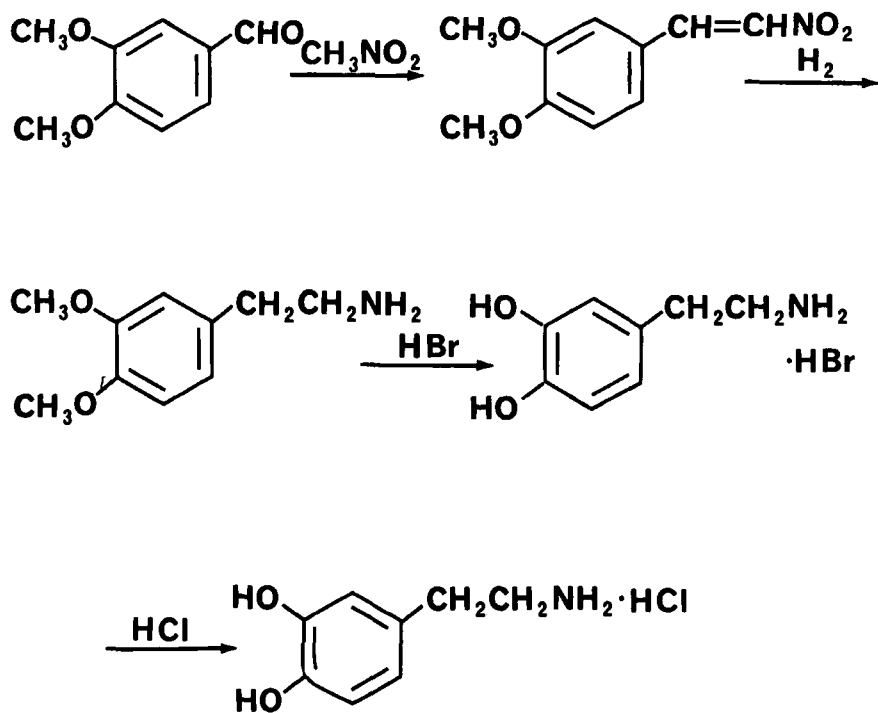


Fig. 5 Synthetic Scheme for Dopamine Hydrochloride

The dimethoxyphenethylamine (known as homoveratrylamine) has been converted to dopamine hydrochloride in one step with pyridine hydrochloride (7).

4. Analysis

4.1 Elemental Analysis

Elemental analysis of a typical dopamine hydrochloride sample is as follows:

Element	% Theoretical	% Found
C	50.67	50.51
H	6.38	6.55
Cl	18.70	---
N	7.39	7.37
O	16.87	---

4.2 Colorimetric Assay

A colorimetric assay for the analysis of dopamine has been adapted from The United States Pharmacopeia Epinephrine Assay (8). "The Photometric Detection of Adrenaline in Pharmaceutical Products" was first described by Doty in 1948 (9).

4.3 Non-aqueous Titration

The purity of dopamine hydrochloride may be assessed by non-aqueous titration with crystal violet as the indicator. The hydrochloride salt is dissolved in glacial acetic acid, mercuric acetate is added to remove the chloride as unionized mercuric chloride, crystal violet is added and the amine is titrated to a green end-point with 0.1 N perchloric acid.

4.4 Thin-layer Chromatography (TLC)

Thin-layer chromatography on silica gel is particularly helpful in assessing the purity of the raw drug. In a solvent system of ethyl acetate-methanol-ammonium hydroxide (85:10:5) all potential methoxyphenethylamine impurities are readily separated and differentiated. The 3-hydroxy-4-methoxyphenethylamine and dopamine fluoresce under short wave ultraviolet light. After spraying with ninhydrin and developing at 110° for 5 minutes dopamine appears as a brown spot ($R_f \approx 0.07$), the 3-hydroxy-4-methoxy analog appears as a yellow spot ($R_f \approx 0.22$), the 3-methoxy-4-hydroxy analog appears as a pink spot ($R_f \approx 0.26$) and the dimethoxy compound appears as a pink spot ($R_f \approx 0.30$).

In a solvent system of n-butanol-glacial acetic acid-water (12:3:5), dopamine has an R_f of approximately 0.6. The compounds may also be visualized by spraying the silica plate with 0.5% iodine in chloroform.

4.5 Gas Chromatography

Because of the high-boiling, polar and oxidizable nature of dopamine, it is not readily amenable to gas chromatography.

4.6 High Performance Liquid Chromatography (HPLC)

No work has been reported for the HPLC analysis of dopamine hydrochloride in pharmaceutical dosage forms as might be expected for a non-patented product. One relevant paper reports the chromatographic behavior of dopamine and related catecholamines in a reverse phase system on the ubiquitous octadecylsilane column (10). The authors found dopamine to be insensitive to pH changes between 2 and 5 with nitric acid but to be retained longer as the pH increased in acetic acid. Dopamine appeared to form ion pairs being retained for longer times with trichloroacetic acid and octylsulfonic acid than with mineral acids.

5. Stability

Dopamine hydrochloride (like other catecholamines) is relatively unstable to heat, light and oxygen. The presence of oxidizing agents or trace metals such as copper or iron also increases the rate of degradation. Properly stored in glass containers the raw drug is stable for three to five years.

Dopamine hydrochloride is generally formulated with 1% sodium bisulfite as an antioxidant. The resultant solution has a pH of about 4 and when properly protected from air, heat and light is stable for three to five years.

The stability and compatibility of dopamine solutions with common intravenous fluids (11) various antibiotics (12) and miscellaneous drugs and additives with which it could be potentially mixed (13) has been reported. Dopamine is unstable at an alkaline pH. Thus dopamine solutions are not stable in sodium bicarbonate but are stable in dextrose, sodium chloride, sodium lactate, lactated Ringer's solution, and mannitol.

6. Analysis of Biological Samples

Because of its rapid metabolism, methods for the routine analysis of dopamine in blood and urine have not been developed. Methods which have been reported are generally concerned with endogenous dopamine and its metabolism rather than the clinical pharmacology and analysis of infused dopamine.

The analysis of ^{14}C -dopamine and its radiolabelled metabolites following an infusion in human subjects has been reported (14).

A novel approach which may be applicable to analysis following a dopamine infusion has recently been developed (15). This method converts dopamine (and other catechols) to its methoxy derivative in the presence of catechol-O-methyltransferase and S-adenosylmethionine- ^3H methyl. The tritiated amines are extracted from plasma with diethyl ether, separated by TLC and measured in a scintillation counter. The method is reported sensitive to 0.1 nmole/L.

7. Metabolism and Excretion

Dopamine metabolism and excretion was studied following intravenous administration of ^{14}C material to six healthy males (14). Dopamine and its metabolites are excreted primarily in the urine; the radioactive dose was quantitatively recovered after 5 days. Approximately 75% of the dose was converted into dopamine-related metabolites. The principal product was 3-methoxy-4-hydroxyphenylacetic acid. Other prominent metabolites were 3-methoxydopamine, 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxyphenylethanol and 3-methoxy-4-hydroxyphenylethanol. The remaining 25% of the infused dopamine was transformed into norepinephrine and appeared in the urine principally as metabolites of norepinephrine.

The conjugates of dopamine and its metabolites were studied in cultured human skin fibroblasts and rat hepatoma cells (16). These studies along with references cited therein indicate the principal dopamine conjugates as the 3-O sulphate, and the 3-O-glucuronide.

8. Acknowledgement

The manuscript was expertly typed by Ms. Martine Bunting.

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ERGONOVINE MALEATE

Van D. Reif

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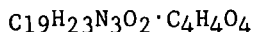
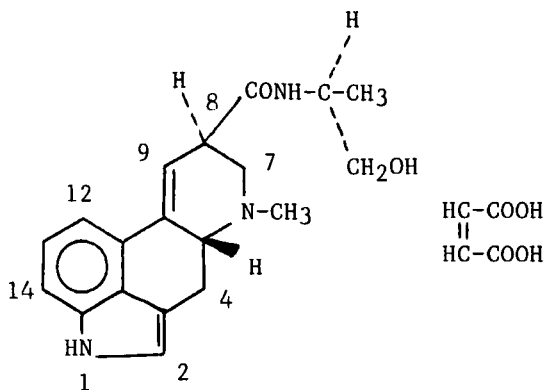
1. Description

1.1 General Classification

Ergonovine is a naturally occurring alkaloid found in ergot (*Claviceps purpurea*). It is classed as one of the water-soluble, amine ergot alkaloids, and is an orally-active oxytocic (1,2). The maleate salt exhibits greater stability than the free base and is the usual form in which the alkaloid is utilized (3).

1.2 Name, Formula, Molecular Weight

The name used by Chemical Abstracts for ergonovine maleate is [8 β (S)]-9,10-didehydro-N-(2-hydroxy-1-methylethyl)-6-methylergoline-8-carboxamide, (Z)-2-butenedioate (1:1) salt. The Chemical Abstracts Registry Number is 129-51-1. Ergometrine, ergostetrine, ergobasine, ergotocine, and D-lysergic acid L-2-propanolamide are other names that have been used for this alkaloid (2,4).



Mol. Wt. = 441.48

1.3 Appearance, Color, Odor

Ergonovine maleate is a white to greyish white, odorless, crystalline powder.

2. Physical Properties

2.1 Infrared Spectra

The infrared spectra of ergonovine maleate in potassium bromide and in mineral oil are given in Figures 1 and 2, respectively. Both were recorded on a Perkin-Elmer 467 Grating Spectrophotometer. The KBr spectrum is similar to that previously published (5). The KBr procedure is also used for an official identity test (6). Structural assignments (7,8) of some of the significant bands are given in Table I. As reported (7), a carbonyl band for the maleate moiety is not distinguished in the KBr dispersion, and is seen only as a shoulder at 1690 cm^{-1} in the mull.

Table I

Infrared Assignments for Ergonovine Maleate
(0.5% KBr Dispersion)

<u>Frequency range (cm^{-1})</u>	<u>Assignment</u>
3260-3500	N-H, O-H stretch
1650	amide C=O stretch
1570	carboxylate annion
1055	primary hydroxyl
750,775	indole C-H

2.2 Ultraviolet Spectra

The ultraviolet spectrum of ergonovine maleate in ethanol is shown in Figure 3. An absorptivity of 20.5 ($E=9160$) was determined for the maximum at 311 nm in alcohol. A similar spectrum is obtained using water as solvent; the absorptivity at the maximum at 311 nm was found to be 18.7 ($E=8260$). The absorptivity values agree with those previously published (9,10).

2.3 Fluorescence and Phosphorescence Spectra

A fluorescence emission spectrum in ethanol is shown in Figure 4. Excitation was at 325 nm. It was obtained on a Perkin-Elmer Model 512 fluorescence spectrophotometer. A similar spectrum was reported by Bowd et al. (10) for ergonovine, and phosphorescence maxima were found at 514,554, and 611 nm at 77°K in ethanol.

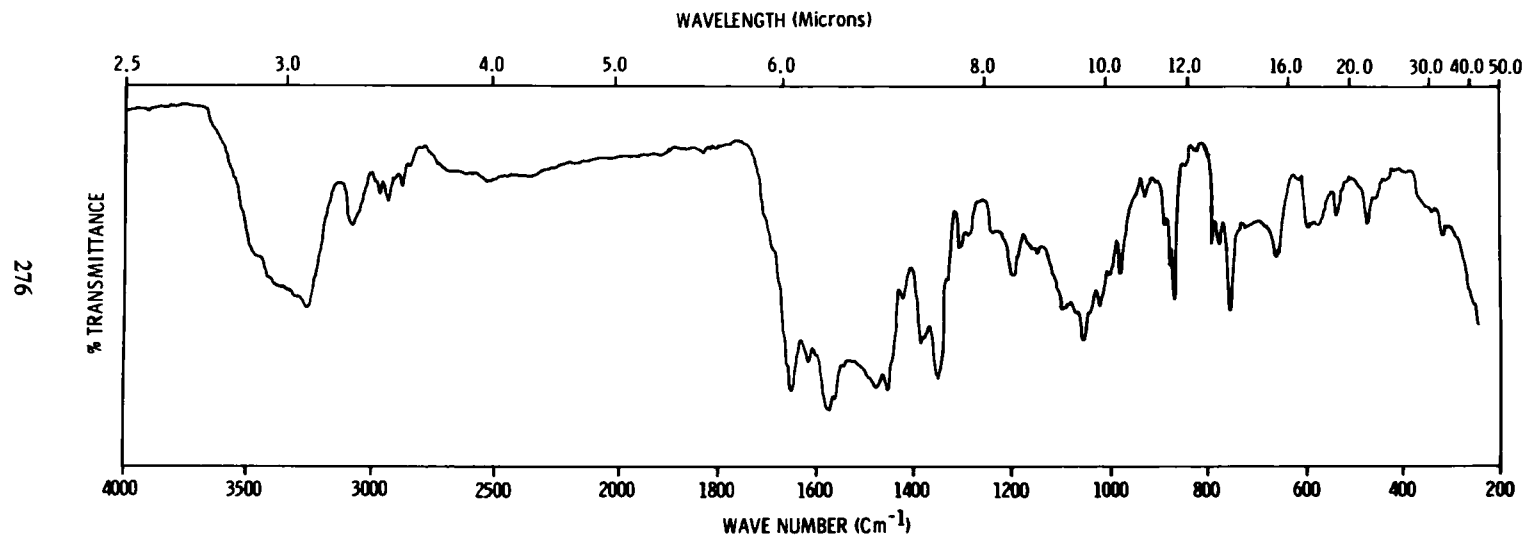


Figure 1 - Infrared Spectrum of Ergonovine Maleate, USP Reference Standard,
Lot L(KBr Pellet)

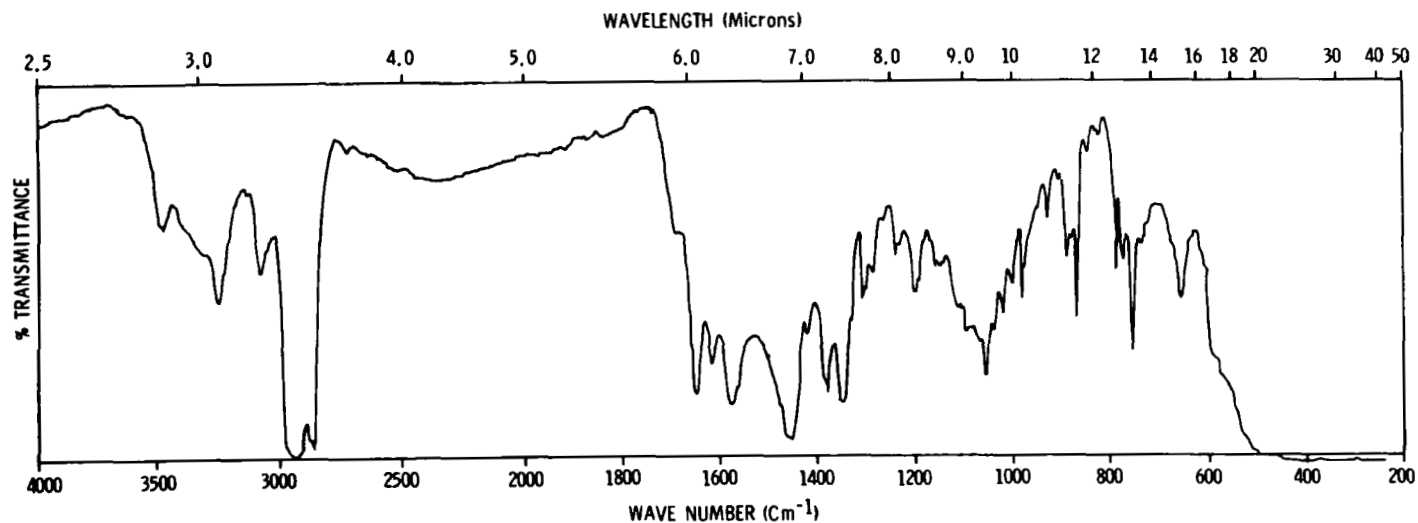


Figure 2 - Infrared Spectrum of Ergonovine Maleate, USP Reference Standard,
Lot L (Mineral Oil Mull)

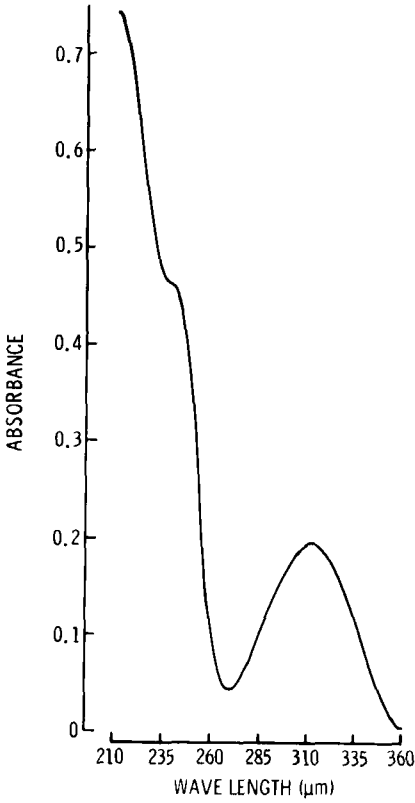


Fig. 3. Ultraviolet Spectrum of Ergonovine Maleate (USP Reference Standard, Lot L). Solvent-Alcohol.

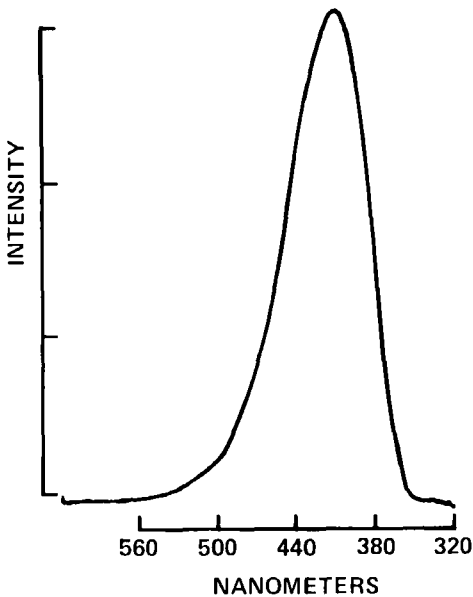


Fig. 4. Fluorescence Emission Spectrum of Ergonovine Maleate, 0.1 mg/ml (USP Reference Standard, Lot L)

2.4 Nuclear Magnetic Resonance Spectra

The proton NMR spectrum of ergonovine maleate (USP Reference Standard, Lot L) in deuterated dimethylsulfoxide (d_6) with tetramethylsilane as internal standard is shown in Figure 5. The spectrum was obtained with a 100 MHz Varian XL-100 spectrometer (11). Spectral assignments are given in Table II. Bands corresponding to side chain hydrogens were identified by irradiation of the side-chain methine hydrogen ($\delta = 3.9$). This resulted in conversion of doublets at 1.12, 3.44, and 8.25 ppm to singlets. The alcohol hydrogen was assigned to the shift at 3.68 ppm because resonance was lost at this shift after D_2O exchange. The shift at 10.98 ppm, assigned to the indole-NH, also showed a partial loss of resonance after D_2O . Similarly, the only resonances above 7.5 ppm for related indoles, 8.5 ppm ($CDCl_3$) for lysergic acid dimethylamide and 11.4 ppm (deuteropyridine) for penniclavine, were attributed to the indole-NH (12,13). The 6-NCH₃, H₈, H₉, and aromatic proton assignments are also similar to those reported for penniclavine and lysergic acid dimethylamide.

Table II

Proton NMR Spectral Assignments for Ergonovine Maleate

<u>Chemical Shift (δ)</u>	<u>Multiplicity</u>	<u>Assignment</u>
1.12	doublet	-CH ₃ (side chain)
3.10	singlet	6-NCH ₃
3.44	doublet	C-CH ₂ O
3.68	singlet	C-OH
3.9	multiplet	NCH-C
4.30	multiplet	α H-8
6.14	singlet	maleate C-H
6.57	sharp multiplet	H-9
7.6-7.4	multiplet	H-12, H-13, H-14, H-2
8.25	doublet	$\begin{array}{c} O \\ \\ CNH \end{array}$
10.98	singlet	indole-NH

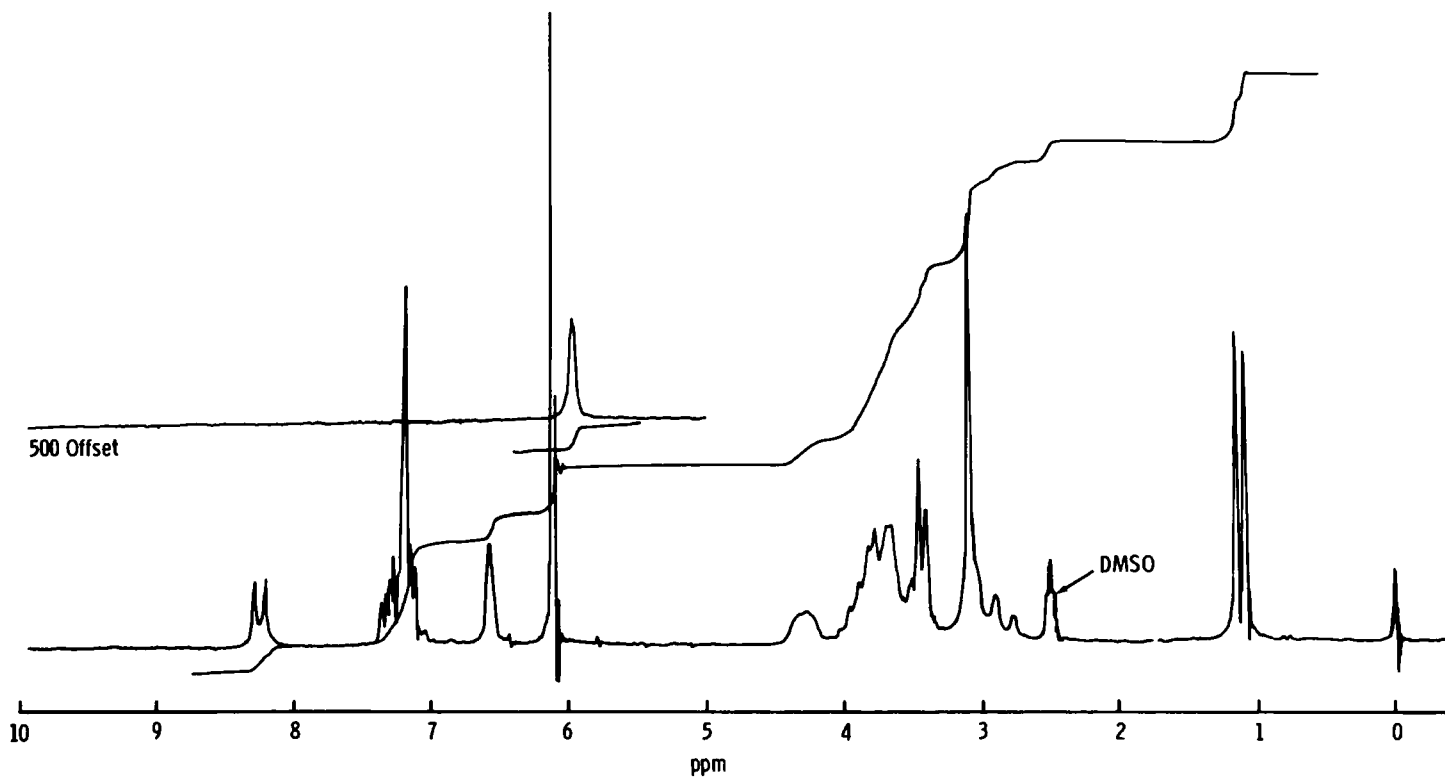


Figure 5 - ^1H NMR Spectrum of Ergonovine Maleate (USP Reference Standard, Lot L) in Deuterodimethylsulfoxide

The ^{13}C NMR spectrum of ergonovine maleate (USP Reference Standard, Lot L) in deuterated dimethylsulfoxide is shown in Figure 6. It was recorded on a Varian FT-80-A spectrometer at ambient temperature (11). The spectral assignments are given in Table III. The chemical shifts are similar to those reported by Bach *et al.* (14) for ergonovine.

Table III

^{13}C NMR Assignments for Ergonovine Maleate

<u>Carbon</u>	<u>ppm</u>
-CO ₂ H	169.3
-CON	168.1
HC=CH	136.2
C-10	134.6
C-15	131.8
C-16	126.0
C-11	125.7
C-13	123.3
C-9	121.0
C-2	120.5
C-14	112.5
C-12	111.6
C-3	106.8
OCH ₂	65.0
C-5	61.8
C-7	53.6
HN-CH	47.7
N-CH ₃	41.7
C-8	40.8
C-4	24.7
-CH ₃ (side chain)	17.7

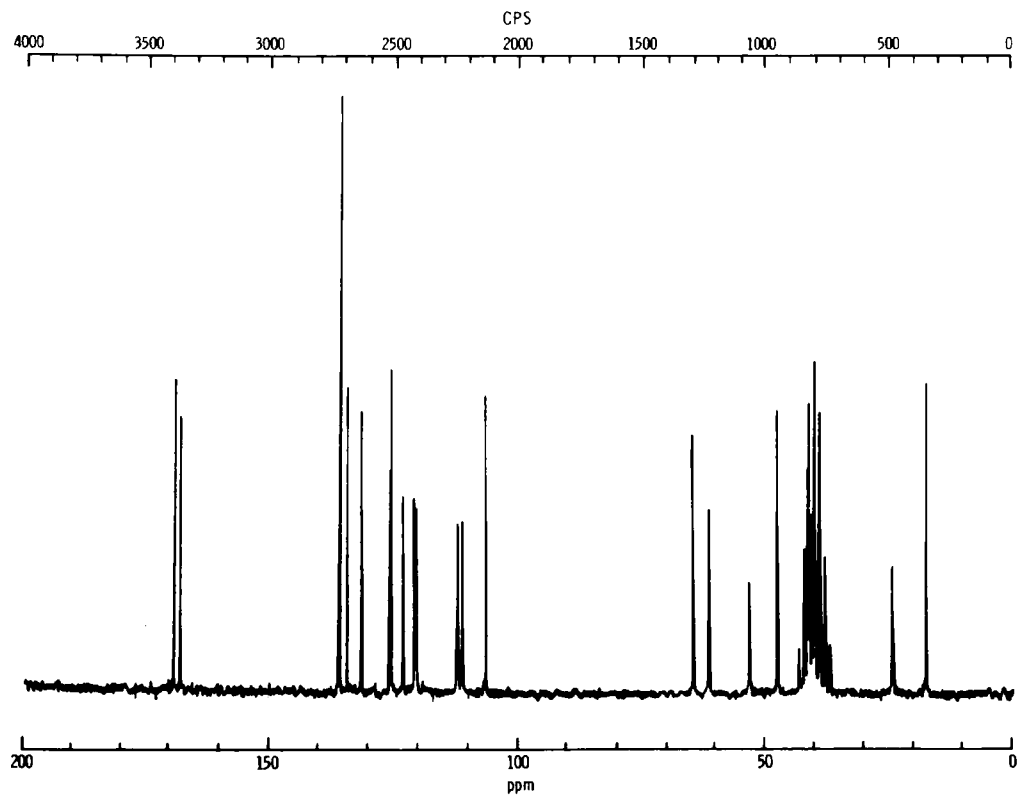


Figure 6 - ^{13}C NMR Spectrum of Ergonovine Maleate (USP Reference Standard, Lot L) in Deuterodimethylsulfoxide

2.5 Mass Spectrum

The mass spectrum of ergonovine maleate was obtained with a Kratos DS-50S Data System coupled with a MS-902 double focusing, high resolution mass spectrometer (15). The ionizing electron beam energy was at 70eV and the probe temperature was 250°. A line graph of the mass spectrum is shown in Figure 7 and identification of the pertinent masses are presented in Figure 8. The retro-Diels-Alder reaction fragments at m/e 282 and 196 and the fragments at m/e 220-223 were postulated by Bellman (16) for other lysergic acid analogs. The identity of fragments at m/e 167 and 154 have been postulated for other ergot alkaloids (17,18).

2.6 Differential Scanning Calorimetry

The DSC thermogram (19) of ergonovine maleate (USP Reference Standard, Lot L) is shown in Figure 9. The thermogram was obtained at a heating rate of 10°/min in a nitrogen atmosphere using a Perkin-Elmer DSC-2. No endotherms or exotherms were observed other than that associated with the decomposition melt.

2.7 Melting Range

The melting range must be taken under a protective environment to limit decomposition. The following melting point temperatures have been reported for ergonovine maleate:

<u>°C</u>	<u>Reference</u>
167 (with decomposition)	4
180-190 (in paraffin)	20
187-191 (silicone oil)	21
186-196 (under nitrogen)	21
185-190 (sealed capillary)	21

2.8 Crystal Properties

The X-ray diffraction pattern of ergonovine maleate (USP Reference Standard, Lot L) as obtained with a Philips diffractometer using CuK α radiation (19), is presented in Figure 10. The calculated "d" spacings are listed in Table IV.

2.9 Solubility

The following solubilities have been reported for ergonovine maleate (4,22):

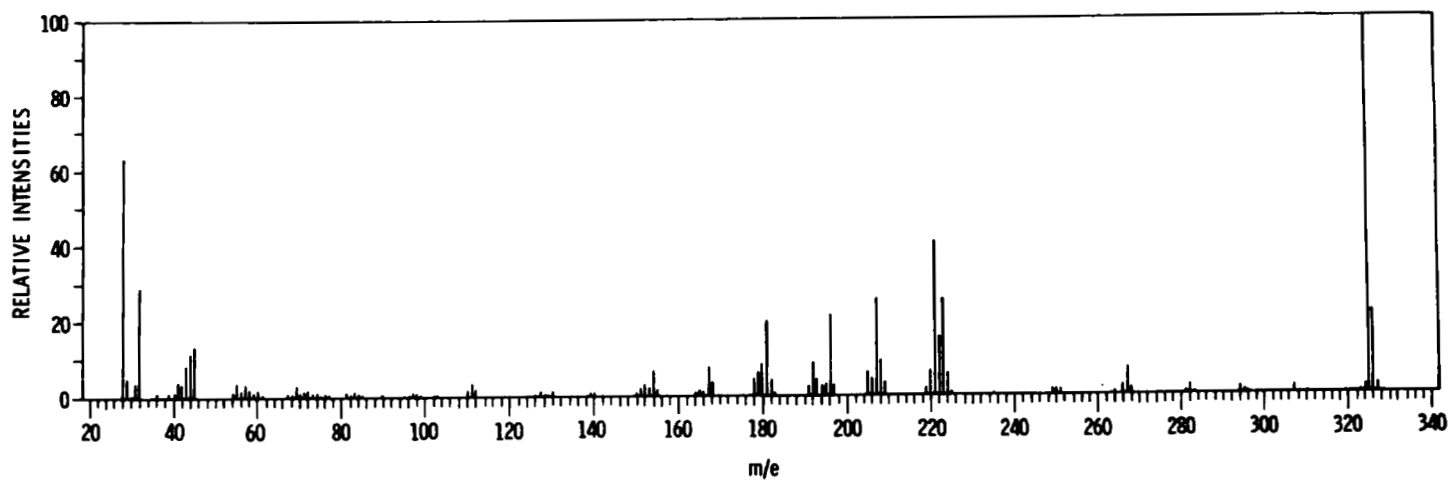


Figure 7 - Mass Spectrum of Ergonovine Maleate (USP Reference Standard, Lot L)

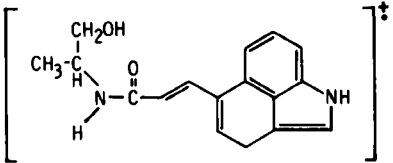
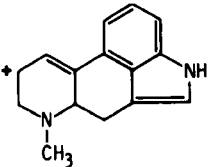
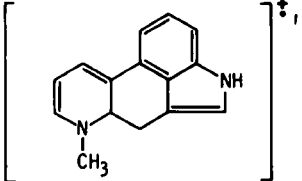
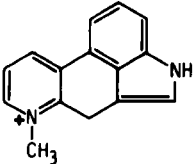
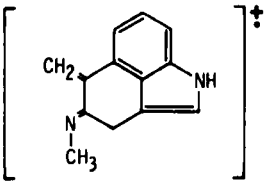
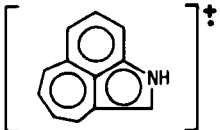
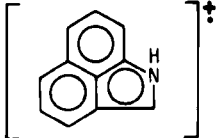
<u>m/e</u>	<u>ASSIGNMENT</u>	<u>RELATIVE INTENSITY</u>
325	m^+	100
310	$m^+ - CH_3$	0.4
307	$m^+ - H_2O$	2.2
294	$m^+ - CH_2OH$	2.0
282		2.8
223		25.5
222		40.9
221		6.8
196		21.4
167		7.3
154		6.7

Figure 8 - Mass Spectrum Fragments

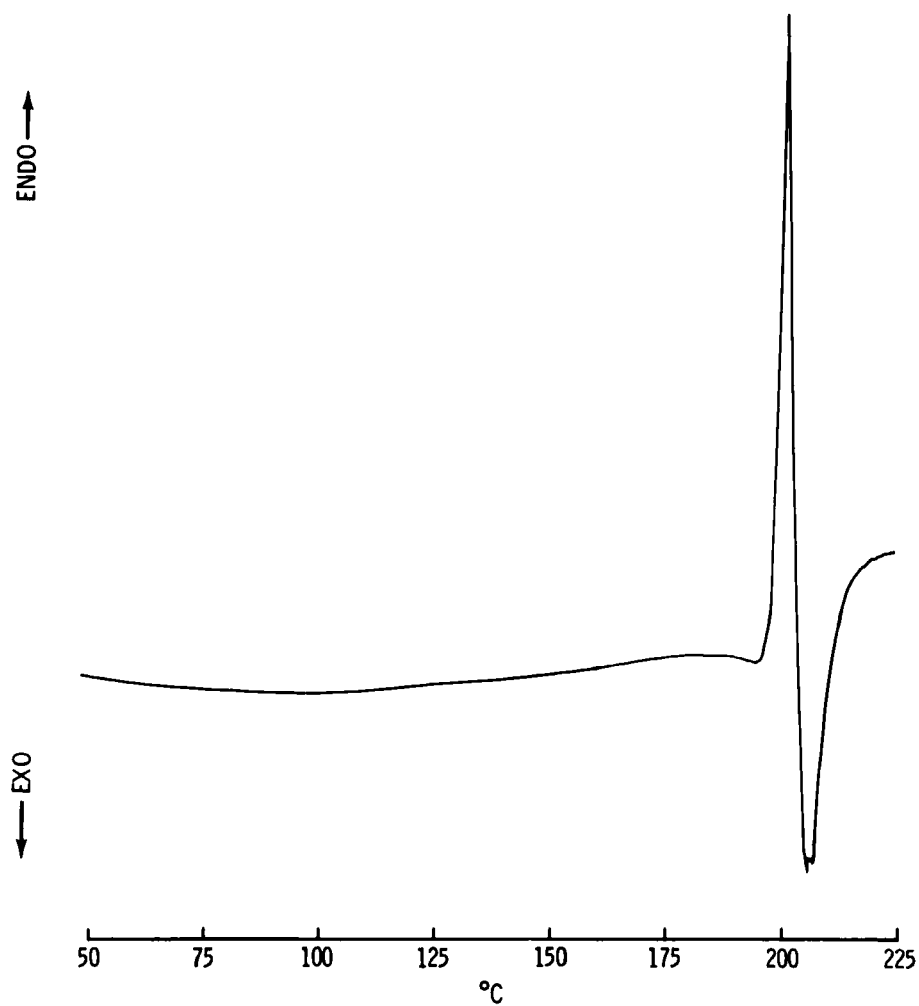


Fig. 9. Differential Thermal Analysis Spectrum of Ergonovine Maleate (USP Reference Standard, Lot L)

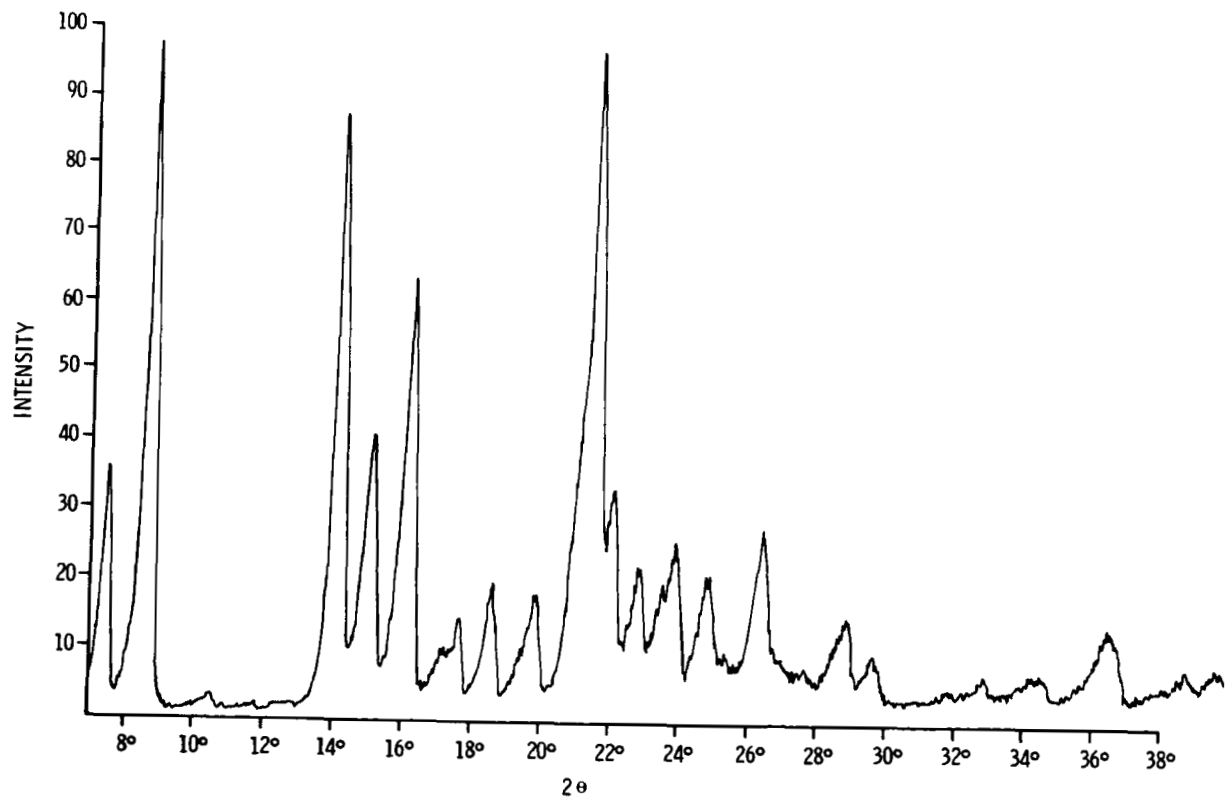


Figure 10 - X-ray Diffraction Pattern of Ergonovine Maleate (USP Reference Standard, Lot L)

Table IV
X-Ray Powder Diffraction Pattern

<u>2θ</u>	<u>d (Å)</u>	<u>I/I₀</u>
39.6	2.28	4
38.7	2.33	4
36.5	2.46	11
34.4	2.61	4
32.8	2.73	4
29.6	3.02	7
28.9	3.09	13
26.4	3.38	26
24.8	3.59	19
23.9	3.72	24
22.9	3.88	20
22.1	4.02	32
21.6	4.11	98
19.9	4.46	16
18.6	4.77	18
17.7	5.01	13
16.2	5.47	64
15.1	5.87	40
14.2	6.24	89
8.8	10.00	100
7.5	12.00	36

<u>Solvent</u>	<u>Solubility (mg/ml)</u>
water	28
alcohol	8
chloroform	nearly insoluble (<0.1)
ether	nearly insoluble (<0.1)

2.10 Optical Rotation

The following optical rotations were reported (23) for ergonovine maleate:

$$[\alpha]_D^{20} = +53 \text{ (C=1.0 in water)}$$

$$[\alpha]_{578}^{20} = +57 \text{ (C=1.0 in water)}$$

$$[\alpha]_{546}^{20} = +74 \text{ (C=1.0 in water)}$$

$$[\alpha]_{436}^{20} = +252 \text{ (C=1.0 in water)}$$

USP material (6) meets the specification,

$$[\alpha]_D^{25} = +51 \text{ to } +56 \text{ (C=0.5 in water)}.$$

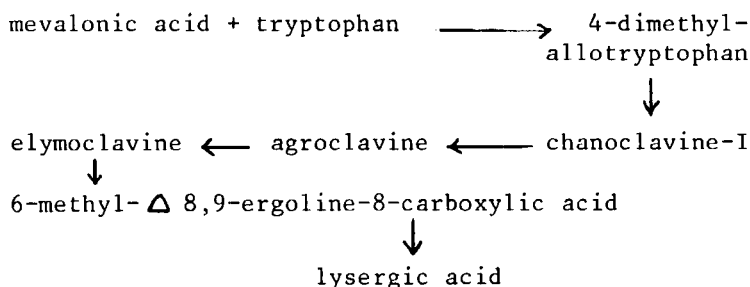
2.11 Circular Dichroism-Configuration

A circular dichroism spectrum was reported (24) for ergonovine maleate. Maxima were at approximately 218 and 318 nm, and a minimum was at 248 nm. Other isomers containing the D-lysergic acid moiety gave similar spectra; a compound containing the L-lysergic acid moiety showed a reversed effect. This is consistent with the positive Cotton above 300 nm reported for D-lysergic acid (25). The rotatory dispersive effects are due to the configuration at C-5; the configuration at C-8 has only an additive or subtractive effect. In the case of D-lysergic acid, and therefore, ergonovine, the hydrogen at C-5 is in the β position (25) and the C-5 center is in the S configuration (26).

3. Synthesis

3.1 Biosynthesis

Ergonovine was originally isolated from ergot extracts (2,27-29). It has since been biosynthesized in cultures of Claviceps species (30-35), and has been isolated from seeds of Ipomoea violacea (36,37) and Argyrea nervosa (38). The following pathway has been constructed (39-44) for biosynthesis of the ergoline skeleton by Claviceps species:



The final step to produce ergonovine was shown to involve incorporation of alanine with lysergic acid (44).

3.2 Chemical Synthesis

Various semi-synthetic procedures for ergonovine maleate involve addition of the alkyl side chain to lysergic acid. The D-lysergic acid starting material is obtained by alkaline hydrolysis of an ergot alkaloid fraction of biosynthetic origin (45). A relatively high-yield sequence is shown in Figure 11. Lithium lysergate is reacted with a sulfur trioxide-dimethylformamide complex at 0°C. L-2-amino-1-propanol is added, and the product is isolated after addition of water and treatment with maleic acid. Procedures for formation of the amide bond using lysergic acid chloride (46) or phosgene (47) have been patented. A complete fifteen-step synthesis of the D-lysergic acid starting material from 3 β -carboxyethylindole has also been reported (48).

4. Stability

The maleate salt shows improved stability over ergonovine base, but the salt also oxidizes and darkens in the presence of oxygen (3). The stability of ergonovine solutions has been improved by the use of antioxidants such as ascorbic acid (49), methionine, and α -mercaptopropionylglycine (50). The known degradation products are shown in Figure 12. Heat treatment of ergonovine (a dextrorotatory isomer) under acid or alkaline conditions causes a reversible isomerization at the 8-position to form the dextrorotatory ergonovinine, also known as isoergonovine. More stringent acid or base treatment eventually results in hydrolysis to lysergic acid, isolysergic acid, and 2-amino-1-propanol (51,52). When ergonovine is refluxed in the

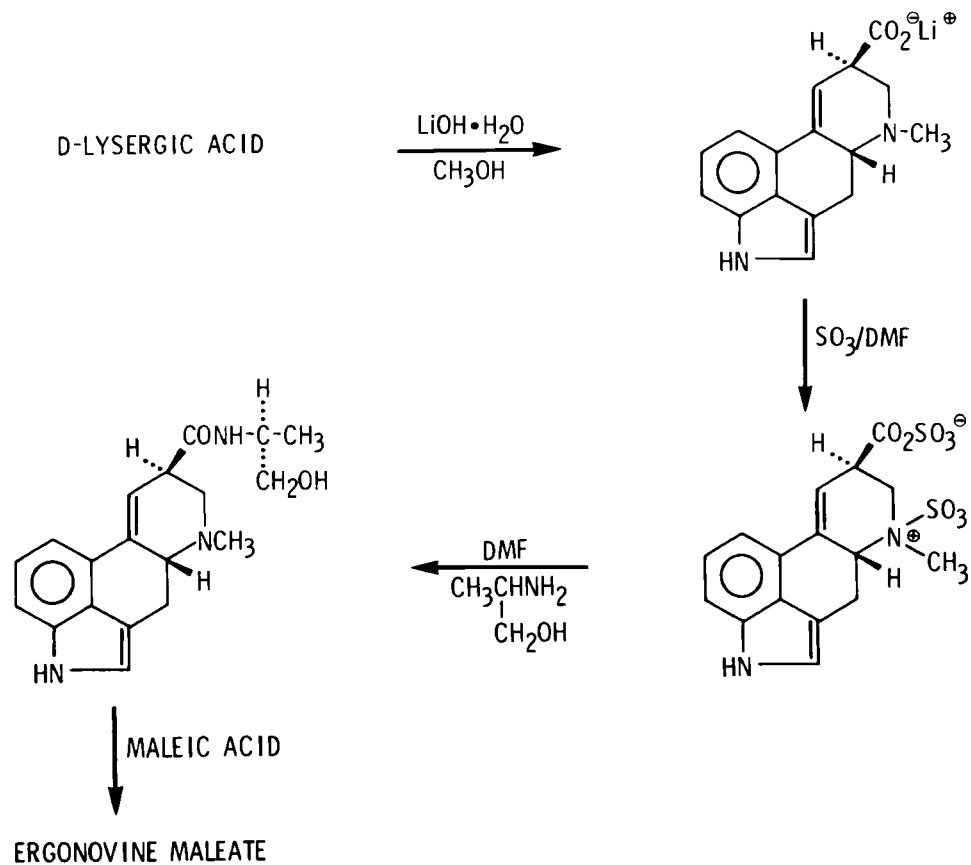


Figure 11 - Synthesis of Ergonovine Maleate from Lysergic Acid

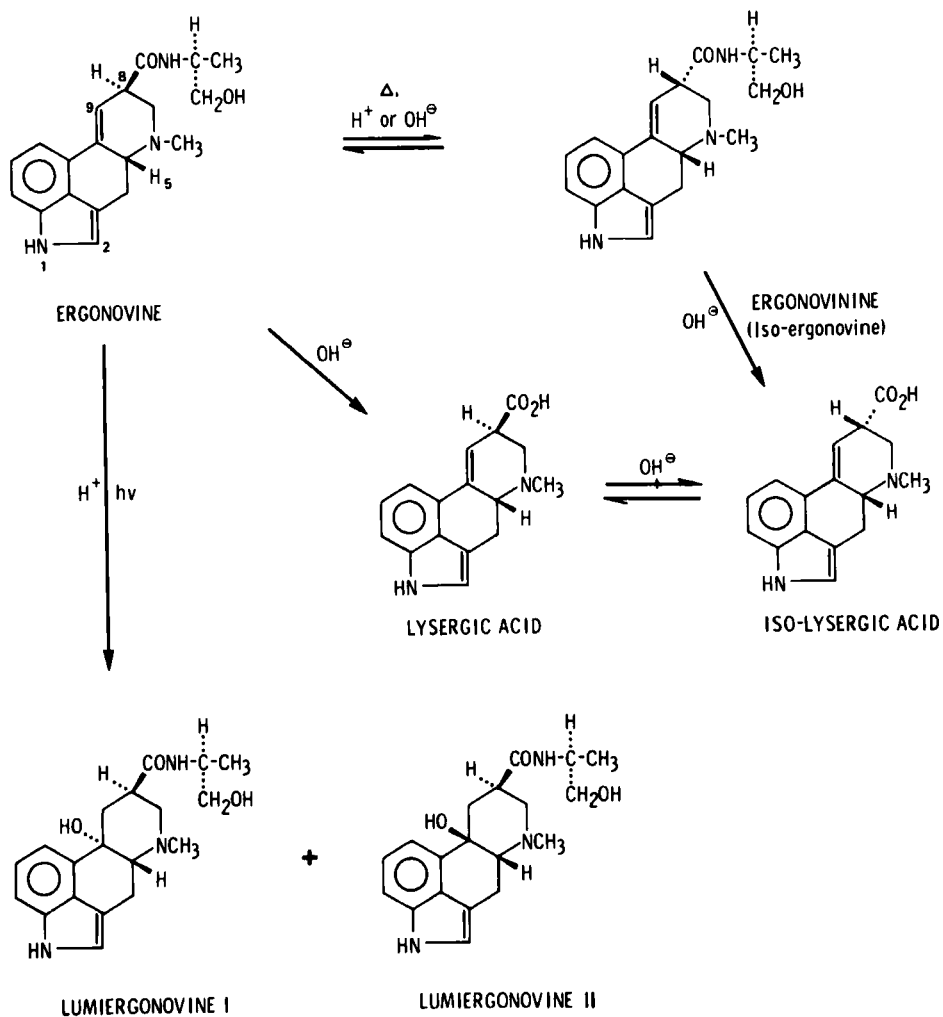


Figure 12 - Degradation of Ergonovine

presence of amines (53), the centers at C-8 and C-5 are both racemized to give mixtures of ergonovine, ergonovine, L-lysergic acid L-2-propanolamide and L-isolysergic acid L-2-propanolamide. When acidic solutions of ergonovine are irradiated, the 9,10 double bond is hydrated to form lumiergonovine I and small quantities of lumiergonovine II (54-56). Degradation of the ergonovine indole ring has been indicated by reports (57) of unidentified fluorescent degradation products which do not react with p-dimethylaminobenzaldehyde.

5. Identification

Alternately known as Van Urk's (58) or Erhlich's (59) reagent, p-dimethylaminobenzaldehyde has had widespread use in ergonovine identity tests (59-61) and chromatographic detection sprays (6,60,62). Indoles give a blue color with this reagent (61); phenols and amines may also react, usually to give other colors (58,59). Color reactions may also be produced with aldehydes such as vanillin, piperonal, and paraldehyde (63). Iodinated potassium iodide (brown flocculent) and ferric chloride in phosphoric acid at 80° (blue-violet color) are two additional identity test reagents (60).

6. Methods of Analysis

6.1 Elemental Analysis

The elemental analysis of ergonovine maleate (USP Reference Standard, Lot L) is presented below.

<u>Element</u>	<u>% Calculated</u>	<u>% Reported (64)</u>
C	62.57	62.55
H	6.17	6.03
N	9.52	9.38

6.2 Direct Spectrophotometric Analysis

Bayer (65) described an ultraviolet spectrophotometric method for ergonovine maleate tablets. The tablets are extracted with 1% tartaric acid and the absorbance is measured at 317 nm.

6.3 Colorimetric Analysis

Official assays for ergonovine maleate drug substance, tablets, and injection (6,60) employ the p-di-methylaminobenzaldehyde reagent. Drug substance or injection samples are dissolved in water; tablet samples are dissolved in aqueous tartaric acid-benzalkonium chloride. The reagent is dissolved in aqueous sulfuric acid-ferric chloride. After color development, absorbances are read at 555 nm. Light or hydrogen peroxide (63,66,67) have also been used in place of ferric chloride to catalyze color development for quantitation. A similar quantitative procedure using sodium nitrite to catalyze color formation was shown to have the advantages of speed, sensitivity, and color stability (62).

The specificity of the reaction for indoles under several conditions was studied by Kupfer (61). The specificity was very dependent on the particular conditions employed and the development times. In general, for indoles to be reactive, an unsubstituted 2- or 3- position was required. The mechanism proposed by Pohm (68) involved attachment of one aldehyde molecule at the 2-positions of two indole molecules with release of water.

An assay for ergonovine maleate tablets using metaldehyde to produce a color at 550 nm has been published (69). A colorimetric assay for tablets using ion-pairing with bromocresol purple was reported (70).

6.4 Titrimetric Analysis

Ergonovine maleate can be determined titrimetrically in glacial acetic acid with a 0.05 N perchloric acid titrant and crystal violet indicator (71,72). The NF XIV drug substance assay (73) is similar, except that acetic anhydride is added to the system. Ergonovine was determined in mixtures after thin-layer chromatography with chloroform-methanol (97:3) on alumina. Spots were eluted with chloroform, acetone was added, and the mixture was titrated with 0.005 N perchloric acid in methanol (74). The maleate moiety has been determined with 0.01 N potassium methoxide titrant, thymol blue indicator, and pyridine as solvent (71).

6.5 Automated Analysis

Kirchhoefer and Wells (75) developed automated methods for ergonovine maleate tablets and injections. Samples were dissolved in pH 6.0 tartrate buffer. For colorimetric quantitation, the sample stream was made basic and extracted with n-butanol. The extract was mixed with p-dimethylaminobenzaldehyde reagent, and after reaction, the stream was mixed with sodium nitrite for determination at 550 nm. For fluorometric detection the sample stream was diluted with tartrate buffer. Excitation was at 325 nm and the emission was read at 432 nm. The methods were tested for interference from common tablet and injection excipients.

Robertson et al. (76) reported a general procedure for basic drugs in which ergonovine was included. Bromthymol blue solution was mixed with an aqueous buffered sample stream. The ergonovine-dye complex was extracted into chloroform and determined at 410 nm.

6.6 Fluorometric Analysis

Fluorometry has been used to determine ergonovine maleate in tablets and injections in an automated procedure (75) (see section 6.5), and after column chromatography (77) (see section 6.73). Samples were read in 0.1 M tartaric acid buffer with excitation and emission at 325 and 432 nm, respectively.

6.7 Chromatographic Analysis

6.71 Thin-Layer Chromatography

The thin-layer chromatographic systems used to analyze ergonovine and ergonovine maleate are listed in Table V. The applications, means of quantitation or detection, and procedure specificity are listed when this information was supplied in the literature sources. The last five systems employ ion-exchange layers.

6.72 Paper Chromatography

Paper chromatographic systems for ergonovine and ergonovine maleate are given in Table VI. Applications and detection agents are also listed. Sprince (100) described a variation of the Ehrlich reagent which gave rapid, long-lasting color development for paper chromatography. A p-dimethylaminobenzaldehyde in HCl spray was followed by a 1% sodium nitrite spray.

6.73 Column Chromatography

The column chromatographic methods that have been used to analyze ergonovine maleate or ergonovine base are described in Table VII.

6.74 Gas Chromatography

Sondack (103) analyzed ergonovine maleate in tablets and injectables after derivatization with N-trimethylsilyldiethylamine and N-trimethylsilylimidazole. A 1% OV-1 on Gas Chrom Q column at 260° was used. The separation of ergonovine and lumiergonovine I from ergonovine was demonstrated.

6.75 High-Performance Liquid Chromatography

High-Performance Liquid Chromatographic systems for ergonovine and ergonovine maleate are listed in Table VIII, along with applications and specificity information.

6.76 Electrophoresis

The electrophoresis of ergonovine on cellulose thin layers at 500V and 3000V was reported (112). The following electrolytes were found to be the most suitable: formic acid-acetic acid-water (26:120:1000), ammonia-water (2:98), and ammonia-water (1:9). A high voltage paper electrophoretic separation of ergonovine from its diastereomer, D-lysergic acid D-propanolamide, was reported (113).

6.8 Radiochemical Procedures

A radioimmuno assay for ergonovine and other ergot alkaloids in plasma was reported (114). Detection was at the picomole level from 4 ml of plasma. The antisera, produced by a conjugate of lysergic acid with human serum albumin, reacted with lysergic acid derivatives, but not with tryptophan. An antibody produced by a mixed anhydride reaction of lysergic acid with bovine serum albumin reacted with simple lysergic acid derivatives and some clavines (115). A similar coupling using a Mannich reaction yielded an antibody specific for lysergic acid.

7. Metabolism

Two metabolites of ergonovine, identified as the β -glucuronides of 12-hydroxyergonovine and 12-hydroxy-ergonovine, were excreted in the bile of rats (99). Two other metabolites were tentatively identified as glucuronides of ergonovine and ergonovine. An earlier report (116) demonstrated the formation of two unidentified polar metabolites of ergonovine in rats.

Table V

Thin-Layer Chromatography Systems

<u>Solvent/Plate</u>	<u>R_f x 100</u>	<u>Application</u> <u>(Quantitation/Detection)</u>	<u>Separation of</u> <u>Ergonovine from:</u>	<u>Ref.</u>
Toluene-Butanol (4N HCl saturated) (6:4)/silica gel	30	Quantitation of ergonovine in ergot mixtures (colorimetric)	Ergonovine, ergot alkaloids	78
Chloroform-Methanol (3:1)/silica gel	-	Quantitation of ergonovine in tablets, injections (<u>in situ</u> fluorometric)	-	79
Chloroform-Methanol (97:3)/alumina	5	Quantitation of ergot mixtures (titrimetric)	Ergotamine, ergocryptine, ergocrystine	74
Ethyl Acetate-n-Heptane- Diethylamine (5:6:0.0005)/ Cellulose-formamide	-	Quantitation of ergot mixtures (<u>in situ</u> fluorometric)	8 Ergot alkaloids	80
Chloroform-Ethanol-Acetone (6:4:4)/silica gel G	23	Ergot alkaloid quantitation (colorimetric)	Ergonovine, 10 ergot alkaloids	81
Chloroform-Ethanol (9:1)/silica gel G	5	Ergot alkaloid quantitation (colorimetric)	Ergonovine, 10 ergot alkaloids	81

Table V (continued)

<u>Solvent/Plate</u>	<u>R_f x 100</u>	<u>Application</u> (<u>Quantitation/Detection</u>)	<u>Separation of</u> <u>Ergonovine from:</u>	<u>Ref.</u>
Ethyl Acetate-DMF-Ethanol (13:1.9:0.1)/silica gel	17	Ergot alkaloid quantitation (colorimetric)	11 Ergot alkaloids	82
Benzene-DMF (13:2)/silica gel	12	Ergot alkaloid quantitation (colorimetric)	11 Ergot alkaloids	82
Methanol-Acetic Acid-Water (2:1:2)/silica gel	30	Ergot alkaloid quantitation (colorimetric)	Ergotamine, agroclavine, chanoclavin	83
Methanol-Water-Tartaric Acid (40:60:1)/silica gel	41	Ergot alkaloid quantitation (colorimetric)	Ergotamine, agroclavine, chanoclavin	83
Benzene-Chloroform-Ethanol (2:4:1)/silica gel	11	Ergot extract quantitation (colorimetric)	8 Ergot alkaloids, ergonovinine	84
Chloroform-Methanol-Water (75:25:3)/silica gel	-	Purity determination and Identity for Ergonovine maleate (visual after p-dimethylaminobenzaldehyde)	-	5
Chloroform-Acetone-Methanol-Triethylamine (15:10:5:1)/silica gel	45	Identification of Impurities in Ergonovine maleate (UV)	Ergonovinine, related alkaloids	24

Table V (continued)

<u>Solvent/Plate</u>	<u>R_f x 100</u>	<u>Application</u> (<u>Quantitation/Detection</u>)	<u>Separation of</u> <u>Ergonovine from:</u>	<u>Ref.</u>
6 Systems/silica gel, alumina, cellulose	-	Ergot Alkaloid Identification (10% CuSO ₄ -2% ammonium hydroxide, 5:1)	Ergonovinine, 5 ergot alkaloids	85
34 Systems /silica gel, alumina	0 to 80	Alkaloid Identification in I. violacea extracts (UV, p-dimethylamino-benzaldehyde + sodium nitrite)	Ergonovinine, LSD, iso-LSD	86
17 Systems/silica gel, alumina, cellulose	0 to 66	Ergot Alkaloid Identification (UV, Ehrlich's Reagent)	14 ergot alkaloids	87
Chloroform-Methanol (9:1)/silica gel GF	10	LSD Identification (Ehrlich's Reagent)	Ergonovinine, 20 ergot alkaloids	88
Chloroform (NH ₃ saturated)-Methanol (18:1)/silica gel GF	13	LSD Identification (Ehrlich's Reagent)	Ergonovinine, 20 ergot alkaloids	88
Trichloroethane-Methanol (9:1)/silica gel	21	LSD Identification (Ehrlich's Reagent)	9 alkaloids	89
Diisopropyl Ether-THF-Toluene-Diethylamine (70:15:15:0.1)/silica gel-formamide	0	Ergot alkaloid separation (UV)	15 ergot alkaloids	90

Table V (continued)

<u>Solvent/Plate</u>	<u>R_f x 100</u>	<u>Application</u> (<u>Quantitation/Detection</u>)	<u>Separation of</u> <u>Ergonovine from:</u>	<u>Ref.</u>
Acetone/0.1M Ammonium Carbonate/ Ethanol (32.5: 67.5:1)/silica gel G	78	Determination of Ergotoxine Alkaloids (vanillin/H ₂ SO ₄)	Ergonovine, 13 ergot alkaloids	91
11 Systems/silica gel, alumina	0-83	methylsergide, methergine detection	methylsergide, methergine	92
Chloroform-Benzene- Methanol (forma- mide saturated, 50:4: 10)/silica gel G	-	Ergot alkaloid identifica- tion (0.5% chlorimino-2,6- dichloroquinone/methanol; 0.3% Congo Red, pH 7; 0.5% quinone/aq. HCl)	4 Ergot alkaloids	93
Acetic Acid-HCl (pH 0.6-2.35)/ alginic acid-cellu- lose, Rexyn 102- cellulose	36-4	Alkaloid identification (6M acetic acid + UV, Dragendorff's)	30 alkaloids	94
1M Acetic Acid in Water-Ethanol (7:3)/Rexyn 102- cellulose	15	Alkaloid identification (6M acetic acid + UV, Dragendorff's)	30 alkaloids	94
1M HCl in Water- Ethanol (1:4)/ Dowex 50-X4-cellulose	21	Alkaloid identification (6M acetic acid + UV, Dragendorff's)	30 alkaloids	94

Table V (concluded)

<u>Solvent/Plate</u>	<u>R_f x 100</u>	<u>Application</u> <u>(Quantitation/Detection)</u>	<u>Separation of</u> <u>Ergonovine from:</u>	<u>Ref.</u>
0.5M Acetate/AG 1-X4 (acetate)-cellulose	21	Alkaloid identification (6M acetic acid + UV, Dragendorff's)	30 alkaloids	95
0.5M Ammonium Acetate/DEAE cellulose	49	Alkaloid identification (6M acetic acid + UV, Dragendorff's)	30 alkaloids	95

Table VI
Paper Chromatography Systems for Ergonovine

<u>Stationary Phase</u>	<u>Mobile</u>	<u>Application</u>	<u>Separations</u>	<u>Detection</u>	<u>Reference</u>
Whatman No. 1 (descending)	n-Butanol-Acetic Acid-Water (4:1:5)	Estimation of ergonovine maleate in ergot ex- tracts and injections	Water-insoluble from water-sol- ble ergot alka- loids, ergometrine from ergometrinine	UV light	96
Whatman No. 3 5% octanol (ascending)	Acetone-5% Aqueous Ammonia (2:3)	determination of ergonovine in ergot extracts	-	p-dimethyl- aminobenzaldehyde	97
A.H. Thomas No. 3677- pH 6 sodium hydroxide- potassium phosphate	n-Butylacetate- Nitromethane- Ethyleneglycol- monoethylether Acetate-Pyridine Water (100:50:50: 8:10)	determination of ergonovine in water-sol- ble ergot extracts	ergonovine from ergonovinine	UV	98
Paper loaded with 10% dimethyl phthalate in chloroform	Formamide-0.1N Potassium Hydroxide (1:4)	limit test for impurities in ergonovine maleate	-	p-dimethylamino- benzaldehyde	60

Table VI (continued)

<u>Stationary Phase</u>	<u>Mobile</u>	<u>Application</u>	<u>Separations</u>	<u>Detection</u>	<u>Reference</u>
Whatman No. 1 or No. 3	Butanol (water saturated)	metabolite iden- tification	Ergonovine from 12-hydroxy- ergonovine, metabolites	UV, Ehrlich's Reagent	99
Whatman No. 1 or No. 3	Butanol (ammonia, water saturated)	metabolite iden- tification	Ergonovine from 12-hydroxy- ergonovine, metabolites	UV, Ehrlich's Reagent	99

Table VII
Column Chromatography for Ergonovine

<u>Packing</u>	<u>Eluent</u>	<u>Application</u>	<u>Separates Ergonovine from</u>	<u>Detection</u>	<u>Ref.</u>
Silica gel	Chloroform-Acetone-Methanol-Triethylamine (15:10:5:1)	Impurity Isolation	Ergometrine, D-LSA-D-2-propranolamide maleate	TLC	24
Sephadex LH-20	96% Ethanol or DMF or Acetone	Plant Extracts	5 Ergot Alkaloids	Van Urk Reagent	101
Sephadex G 25	Water	Plant Extracts	Lysergic Acid	Van Urk Reagent	101
Celite/ Sodium Bicarbonate	Ether	Assay Tablets Injections	-	Fluorescence	77
Celite/ Water	Butanol-Benzene-20% Acetic Acid (1:1:2)	Degraded Extracts	Lumiergonovine I and II	Van Urk Reagent	56
Celite/ Citric Acid	(1)Chloroform (2)Sodium Bicarbonate (3)Chloroform	Powdered Ergot Extracts	Water-Insoluble Ergot Alkaloids	p-Dimethyl-amino benzaldehyde	98

Table VII (continued)

<u>Packing</u>	<u>Eluent</u>	<u>Application</u>	<u>Separates Ergonovine from</u>	<u>Detection</u>	<u>Ref.</u>
Cellulose/ pH 3.0 Citrate- Phosphate	(1)0.1% pyri- dine/ether (2)10% diethyl- amine/ether (3)ether	Ergot Alkaloid Mixtures	Water Insoluble Ergot Alkaloids, Ergonovine	p-Dimethyl- amino benzal- dehyde	102

Table VIII

High-Performance Liquid Chromatography Systems for Ergonovine

<u>Column</u>	<u>Mobile</u>	<u>Application</u>	<u>Ergonovine Separates</u> <u>from</u>	<u>Detection</u>	<u>Ref.</u>
microBondapak C18	Acetonitrile-1% Acetic Acid (1:4)	Injection, Tablet Assay	Ergonovinine, Lysergic Acid	312 nm	104
LiChrosorb RP-2, RP-8 and RP-18	Acetonitrile-0.01M Ammonium Carbonate (2:3)	Identification of Components of Plant Extracts, Fermentation Mixtures	Ergonovinine, 11 Ergot Alkaloids	320 nm	105
LiChrosorb SI-60	n-Hexane-Chloroform-Ethanol (4:4:1)	Identification of Components of Plant Extracts, Fermentation Mixtures	Ergonovinine, 11 Ergot Alkaloids	320 nm	105
Corasil C18	Methanol-0.1% Ammonium Carbonate (3:2)	Assay of Illicit LSD Preparations	Ergot Alkaloids	Fluorometric	107
Corasil II	Acetonitrile-Isopropyl Ether (1:3)	Assay of Illicit LSD Preparations	Ergot Alkaloids	254 nm	108
microBondapak C18	Methanol-Acetic Acid-0.005 M Heptane Sulfonic Acid (40:1:59)	Forensic Mixture Identity	Ergot Alkaloids	254 nm	109

Table VIII (continued)

High-Performance Liquid Chromatography Systems for Ergonovine					
<u>Column</u>	<u>Mobile</u>	<u>Application</u>	<u>Ergonovine Separates</u>	<u>Detection</u>	<u>Ref.</u>
microPak NH ₂	Diethyl Ether-Ethanol (84:12)	Separation of Ergot Mixtures	Ergonovinine, 15 Ergot Alkaloids	310 nm	110
	Diethyl Ether-Isopropanol (3:2)	Separation of Ergot Mixtures	Ergonovinine, 15 Ergot Alkaloids	310 nm	110
	Chloroform-Iso-propanol (9:1)	Separation of Ergot Mixtures	Ergonovinine, 15 Ergot Alkaloids	310 nm	110
LiChrosorb NH ₂	Diethyl Ether-Ethanol (93:7)	Separation of Ergot Mixtures	Ergonovinine, 15 Ergot Alkaloids	310 nm	110
LiChrosorb SI-60	Hexane-Chloroform-Acetonitrile-Methanol (55:20:25:3)	Separation of Ergot Mixtures	Ergonovinine, 10 Ergot Alkaloids	320 nm	111

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FLUFENAMIC ACID

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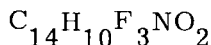
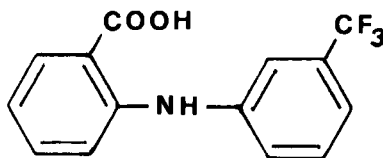
1. DESCRIPTION

1.1. Name

Flufenamic acid is designated by Chemical Abstracts since 1972 as 2-[[3-(trifluoromethyl)phenyl]amino]benzoic acid, whereas before 1972 it was named N-(α,α,α -trifluoro-m-tolyl)anthranilic acid. Other names are: N-(3-trifluoromethylphenyl)anthranilic acid, and 3'-trifluoromethyldiphenylamine-2-carboxylic acid (1).

The CAS Registry Number is 530-78-9.

1.2. Formula, Molecular Weight



Molecular Weight: 281.24

1.3. Elemental Composition

C 59.79%; H 3.58%; F 20.27%; N 4.98%; O 11.32%

1.4. Appearance, Color, Odor, Taste

Pale yellow needles, practically odorless with a slight bitter taste.

2. PHYSICAL PROPERTIES

2.1. Infrared Spectrum

The IR spectrum of flufenamic acid shown in Figure 1 was obtained with a Beckman Mycrolab 620MX spectrophotometer in a KBr pellet containing 0.4 mg of flufenamic acid/100 mg of KBr. Some spectral assignments are given in Table I.

This spectrum is in good accordance with the IR spectrum ($1800\text{--}600\text{ cm}^{-1}$) reported by Kuhnert-Brandstätter et al. (26) for the polymorphic modification III of flufenamic acid (see Section 2.8).

2.2. Nuclear Magnetic Resonance Spectrum

The ^1H NMR spectrum of flufenamic acid is pre-

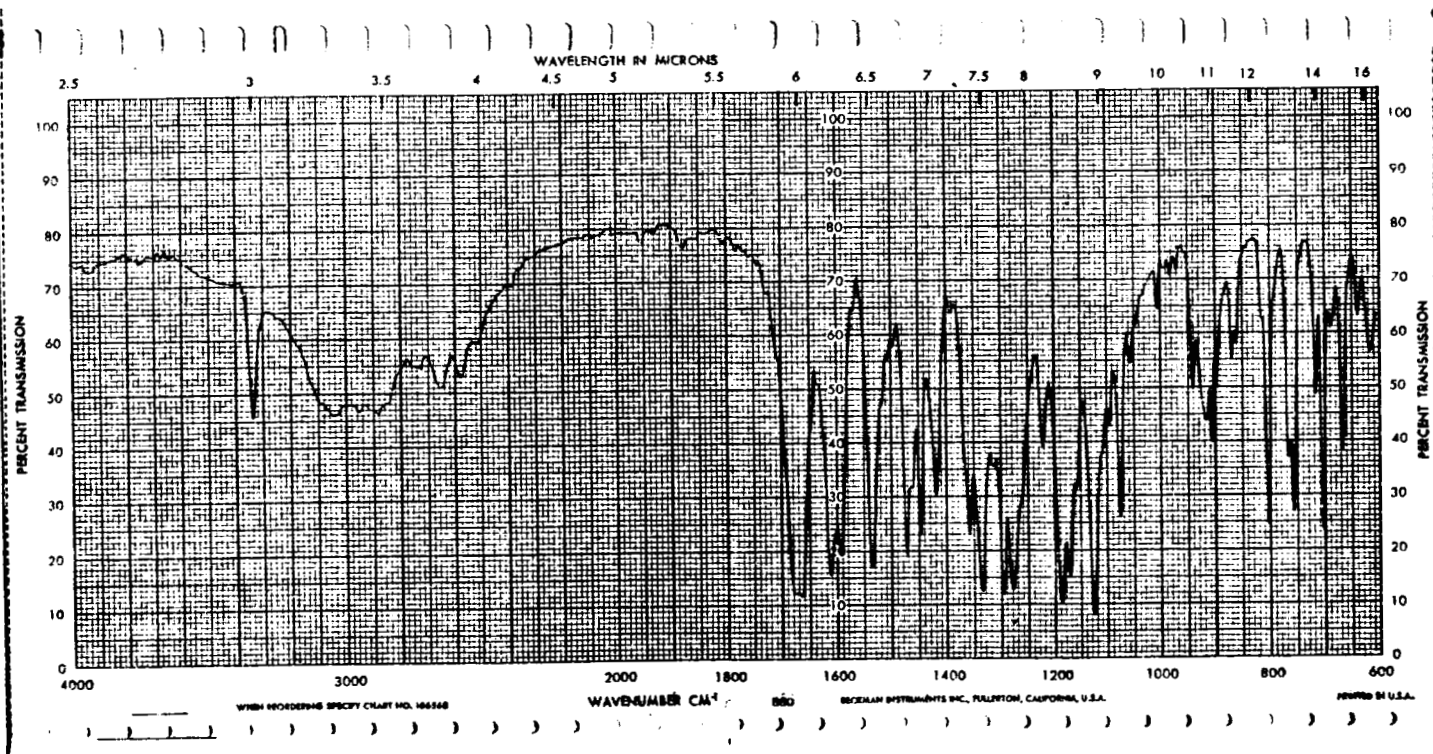


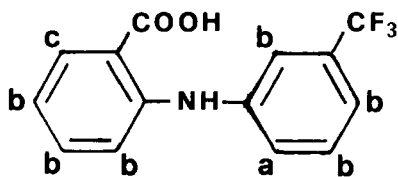
Fig. 1. Infrared Spectrum of Flufenamic Acid

TABLE I
IR Spectral Assignments
of Flufenamic Acid

<u>Wavenumber, cm⁻¹</u>	<u>Vibration Mode</u>
1670	C=O stretching
1612, 1592, 1535	aromatic C=C stretching
1190, 1125	CF ₃ asymm. stretching
803, 768, 758, 703	C-H out of plane bending

sented in Figure 2. The spectrum was recorded on a Varian EM-360 60 MHz spectrometer using a CDCl₃ solution containing tetramethylsilane as an internal standard. The spectral assignments are given in Table II, and are in accordance with the NMR spectrum of etofenamate, which is the 2-(2-hydroxyethoxy)ethyl ester of flufenamic acid, reported by Boltze and Kreisfeld (2) as regards the aromatic protons of the benzene rings.

TABLE II
NMR Spectral Assignments
of Flufenamic Acid



<u>Protons</u>	<u>Chemical Shift, δ</u>	<u>Multiplicity</u>
a	6.60-6.96	multiplet
b	7.05-7.53	multiplet
c	8.05	doublet

2.3. Ultraviolet Spectrum

The UV spectrum of flufenamic acid (Figure 3) was scanned from 400 to 210 nm on a Cary 219 spectro-

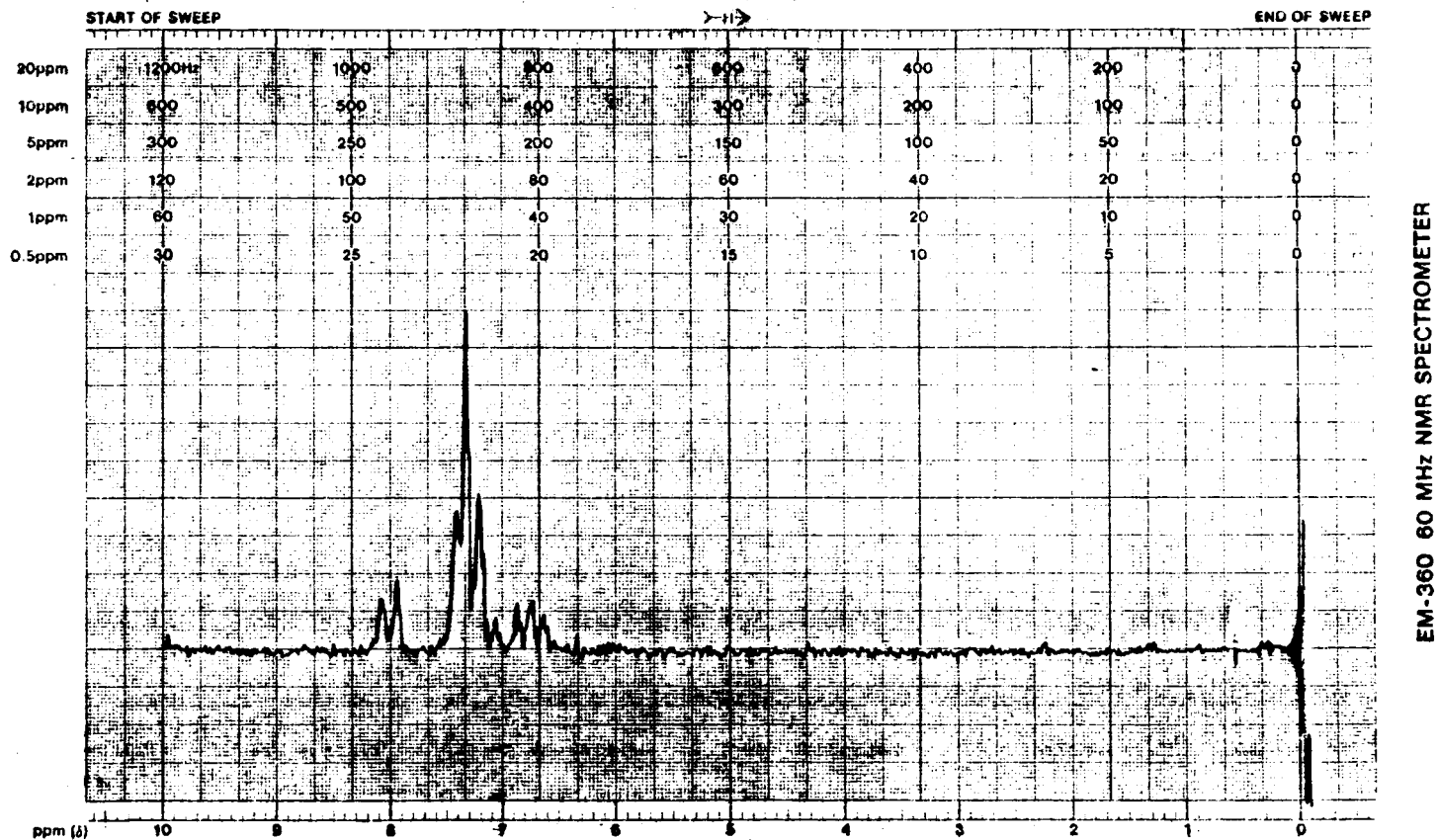


Fig. 2. NMR Spectrum of Flufenamic Acid

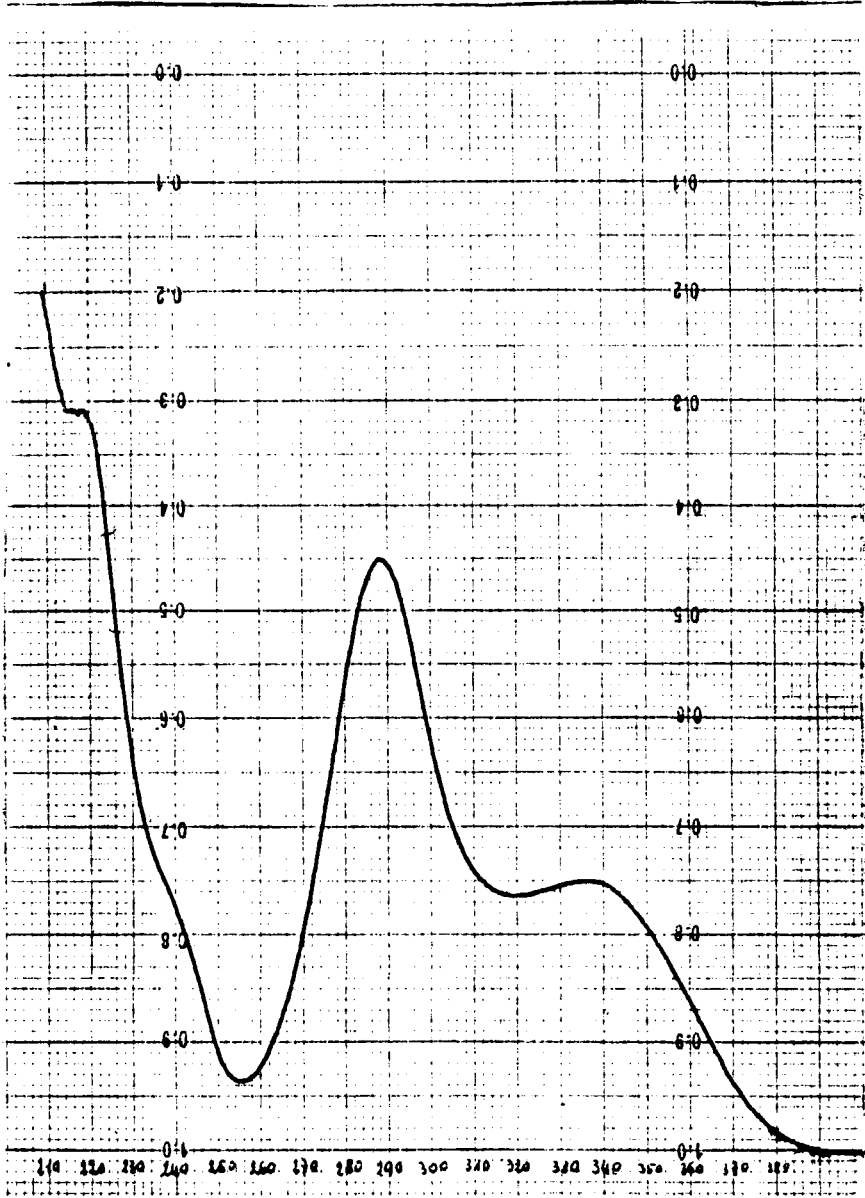


Fig. 3. Ultraviolet Spectrum of Flufenamic Acid

photometer, using a solution of 9.28 μg of flufenamic acid /ml of 95% ethanol. The absorption data are listed in Table III.

TABLE III

UV Absorption Data of Flufenamic Acid in 95% ethanol solution

λ_{max} , nm	$\log \epsilon$	$E_{1\text{ cm}}^{1\%}$
219	4.32	745
238	shoulder	-
288	4.22	590
337	3.88	269

The spectral features observed are substantially in accordance with those previously described in the literature (3, 4, 5, 6, 7, 8). The $\log \epsilon$ vs. wavelength plot of a methanolic solution of flufenamic acid was reported by Unterhalt (3), whereas Ikeda et al. (5) presented the spectrum of the drug in 0.1 M phosphate buffer (pH 7.0). Dell et al. (8) reported λ_{max} values of flufenamic acid and its phenolic metabolites in neutral, acidic and alkaline methanol. In Table IV are listed some absorption data reported in the literature.

TABLE IV

UV Absorption Data of Flufenamic Acid

Solvent	λ_{max} , nm	$\log \epsilon$	$E_{1\text{ cm}}^{1\%}$	Ref.
Methanol	288	4.22	590	3
	340.5	3.89	276	
0.01 N NaOH	289	4.10	448	3
0.1 N NaOH	288	4.18	545	4
0.01 N HCl (in CH_3OH)	287	4.22	590	3
	345	3.94	310	

2. 4. Mass Spectrum

The mass spectrum of flufenamic acid is shown in Figure 4. The spectrum was run on a Hewlett-Packard Mod. 5982A spectrometer with an ionizing energy of 70 eV, interfaced with a Hewlett-Packard Mod. 5934A data-handling system. The computer calculated ion masses and compared their peak intensities to the base peak ($m/e = 263$). This information was then automatically plotted as a series of lines whose heights are proportional to the peak intensities. The molecular ion peak was observed at $m/e = 281$. Some characteristic peaks observed are listed in Table V. The fragmentation pattern observed is

TABLE V

Mass Spectrum of Flufenamic Acid

<u>Mass (m/e)</u>	<u>Species</u>	<u>Abundance %</u>
281	M^+	44.8
263	$M^+ - H_2O$	100.0
235	263-CO	22.8
216	235-F	12.3
166	216-CF ₂	14.9
$(C_6H_4NC_6H_4)$		

consistent with mass spectral data published by Boltze and Kreisfeld (2) for etofenamate and Cotellessa et al. (43) for the methyl ester of flufenamic acid.

2. 5. Fluorescence Spectrum

Flufenamic acid shows native fluorescence in some organic solvents, e.g. dioxan and chloroform, whereas in ethanol fluorescence is too weak to be analytically useful (8, 9). Miller et al. (10) reported that flufenamic acid showed no significant fluorescence at room temperature in acidic, neutral or alkaline ethanol solution, but was strongly fluorescent at low temperature (77°K), presumably because of the virtual abolition of bimolecular quenching in the latter conditions. Dell and Kutschbach (11) investigated the influence on fluorescence intensity of the solvent and the addition of a halogenoacetic acid, as

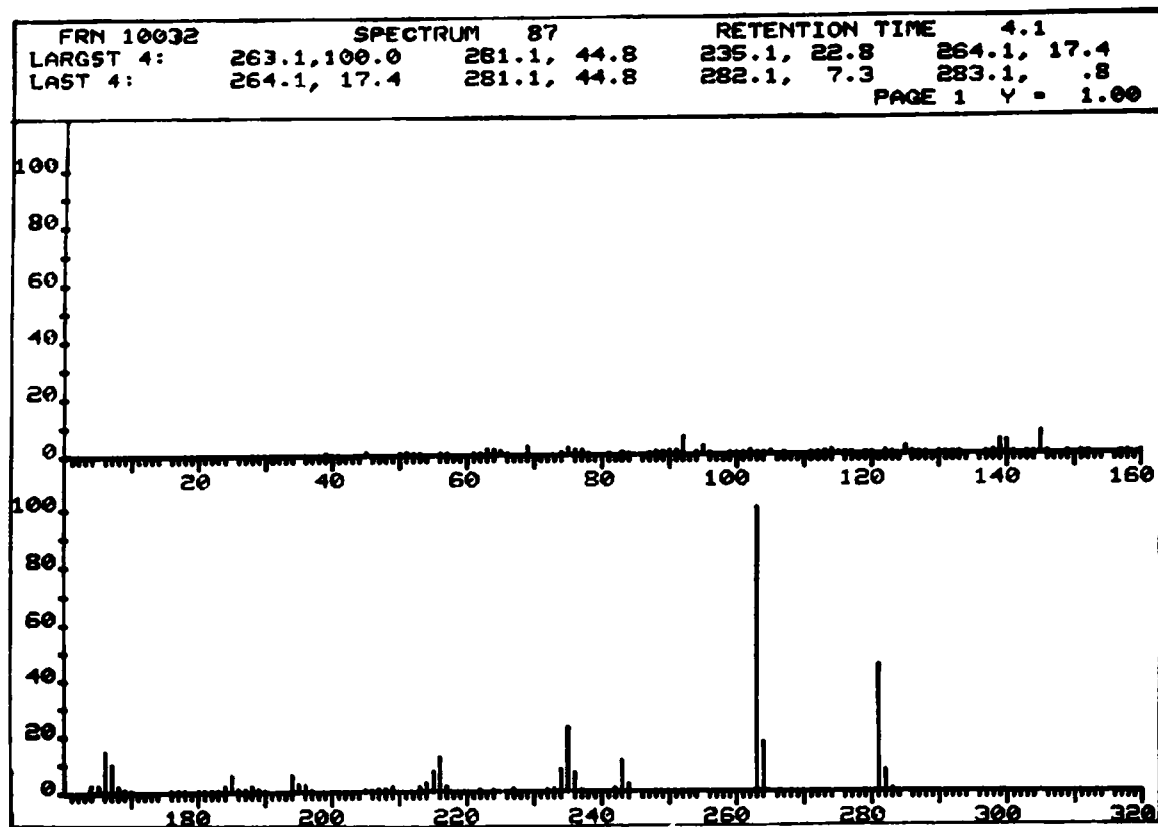


Fig. 4. Mass Spectrum of Flufenamic Acid

well as the influence of a treatment with alkali and/or an oxidant. To obtain a fluorescent solution solvents with a dielectric constant $\epsilon = 0$ are required, and fluorescence intensity is strongly increased by the addition of a halogenoacetic acid with a pK_a value < 1 (e.g. trichloroacetic acid). These findings are the basis for the most common method of fluorometric determination of flufenamic acid (see Section 5.5).

Dell et al. (8) published the fluorescence spectrum of flufenamic acid in a CCl_4 /trichloroacetic acid solution: in this medium flufenamic acid shows the excitation maximum at 372 nm with a subsidiary maximum at 296 nm, and the emission maximum at 445 nm. There is a linear relationship between the fluorescence intensity and the concentration of flufenamic acid up to 10 $\mu g/ml$.

Some fluorescence data reported in the literature for flufenamic acid are listed in Table VI.

TABLE VI
Fluorescence Maxima of Flufenamic Acid
in various solvents

Solvent	Excitation maximum, nm	Emission maximum, nm	Ref.
Methanol	305	400	8
Ethanol ^a	335	510 (420) ^b	10
Dioxan	334	410	9
Chloroform	340	430	9
CCl_4 /TCA	372 (296) ^b	445	8

a : at 77°K.

b : subsidiary maximum.

2.6. Melting range

In Table VII are listed some melting point values reported by various authors for flufenamic acid.

The discrepancies among these values are due to the different polymorphic modifications which can be present in commercial products (see Section 2.8).

TABLE VII

Melting Range of Flufenamic Acid

<u>M. P., °C</u>	<u>Crystallization solvent</u>	<u>Ref.</u>
125	50% ethanol	12
124-125, with resolidification and remelting at 134-136	ligroine	13
127-128	NR	3
132-133	cyclohexane	14, 15
133	NR	16
133-134	NR	2
133-134 or 125-126, resolidifying and remelting at 134-136	NR	17
134.3	ethanol	18
134-136	cyclohexane	19

NR : not reported

2.7. Solubility, Partition Coefficient

Flufenamic acid was reported to be soluble at room temperature in methanol, ethanol, diethyl ether, chloroform, acetone, DMF, and peanut oil (3,4,13). The solubility in water at 22°C is given by Boltze and Kreisfeld (2) as 0.0067 mg/ml. In Table VIII are listed some values of solubility in water at various pH values.

A study by Ghanem et al. (21) indicates that the solubility of flufenamic acid is increased by nonionic surfactants, urea and sodium citrate. The efficiency of the surfactants towards solubilization is in this order: Tween 80 > Brij 99 > Tween 40 > Myrj 53. The effect of urea, amidopyrine, phenazone and paracetamol on the solubility of flufenamic acid and other antirheumatic drugs was studied by Daabis et al. (22).

1-Octanol/water partition coefficient was estimated by Dunn (23) taking advantage of the additive-constitutive na-

TABLE VIII

Solubility of Flufenamic Acid in
Water at various pH Values

pH	Solubility, mg/ml	Ref.
3	0.003 ^a	13
7	1.8 ^a	13
7	1	20
8	4.0 ^a	13

a : at 37°C

ture of log P, as follows:

$$\begin{aligned}\text{Log } P &= \log P_{\text{anthranilic acid}} + \pi_{\text{phenyl}} + \pi_{3\text{-CF}_3} = \\ &= 1.21 + 2.60 + 1.07 = 4.88\end{aligned}$$

Terada et al. (24) have determined the true partition coefficient, P , and the apparent partition coefficient, P' , of flufenamic acid. P was measured by equilibrating a 1-octanol solution of the drug, the initial concentration, C_o , of which was 10^{-3} - 10^{-2} mol/l, with 0.01 N HCl: under this condition, drug molecules exist exclusively as the unionized form. After equilibrium was attained, concentration in the aqueous phase, C_w , was measured spectrophotometrically and P was calculated by the equation: $P = C_o/C_w$, since the concentration change in the organic phase can be neglected for such a highly hydrophobic compound, obtaining $\log P = 5.62$.

P' was determined with the 1-octanol/phosphate buffer (pH 8.0) system, using the equation:

$$P' = \frac{(C_i - C_w) V_w}{C_w V_o}$$

where C and V are the equilibrium concentration and volume of aqueous (subscript w) and organic (subscript o) phases, respectively. C_i is the initial concentration in the aqueous phase. $\log P'$ was found to be 1.74.

Lombardino et al. (25) have also determined the partition coefficient of flufenamic acid with some 1-octanol/buffer systems.

2. 8. Crystal Properties, Polymorphism

Flufenamic acid can exist as several crystalline modifications. Kuhnert-Brandstätter et al. have described five different modifications and have reported their melting points and infrared spectra, as well as the thermogram obtained by differential scanning calorimetry for the first four forms (16, 26).

According to Krc flufenamic acid can exist as at least seven crystalline modifications with different melting points (27). Krc has reported the free energy vs. temperature plot of seven crystalline forms of flufenamic acid. Modifications I, II and III were described in detail in terms of crystal morphology, optical properties, X-ray diffraction powder data and infrared spectra.

Other authors also studied the polymorphism of flufenamic acid. Galdecki et al. (28) investigated the crystallization of the drug from boiling solvents. Burger and Ramberger (29) examined the applicability of some thermodynamic rules to the polymorphic system of flufenamic acid. These rules correlate the heats of transition or fusion, IR spectra and densities of the modifications with their stability behavior. In this study flufenamic acid was investigated mainly by quantitative DSC and qualitative solubility determination (by thermomicroscopy) as well as by IR spectroscopy to differentiate eight crystalline modifications (Table IX). It was pointed out (29) that modifications I, II and III investigated by the various authors are identical, whereas modification IV studied by Kuhnert (26) coincides with modification V by Krc (27), who did not describe Kuhnert's modification V. Since the latter form had the lowest melting point of all eight modifications, it was indicated by Burger and Ramberger as modification VIII.

From the practical point of view, modifications I and III are the most important, because they can be present in the commercial product. The transition point of these two forms is at 42°C: modification III is the stable form

at room temperature (below 42°C), whereas modification I is the stable form above 42°C (27). Modification III was obtained by Burger and Ramberger by stirring for 12 hours at 20°C a xylene suspension of a commercial product formed by modification I (29).

TABLE IX

Melting Points of crystalline Modifications of Flufenamic Acid

<u>Modification</u>	<u>M. P., °C</u>	<u>Ref.</u>
I	133	16, 26
	134	27, 29
II	128	16, 26, 27, 29
III	125	16, 26
	126	29
	126.5	27
IV	124	27
V ^a	122	16, 26, 29
	122.5	27
VI	120	27
VII	118	27
VIII ^b	100-110	26
	108 ± 5	29

a : This modification was indicated as IV in the papers 16 and 26.

b : This modification was indicated as V in the papers 16 and 26.

2.9. Dissociation Constant

The pK_a of flufenamic acid was reported to be 3.9 by Aguiar and Fifelski (20) and 4.5 by Frey and El-Sayed (30). Terada et al. (24) have found a value of 3.85 using the pH-dependent solubility method (31); this value is considerably different from the corresponding

value obtained by potentiometric titration in 5-10% aqueous acetone (32). In Table X are listed some pKa values obtained by potentiometric titration in various aqueous solvent systems.

TABLE X
pKa Values of Flufenamic Acid
obtained by Potentiometric Titration

<u>Solvent</u>	<u>pKa</u>	<u>Ref.</u>
Water	7.5	33
75% Aqueous methanol	5.75	3
50% Aqueous ethanol	5.94	33
80% Aqueous 2-methoxy- ethanol	6.0	33
Dioxane:water (2:1)	6.8	25

3. SYNTHESIS

Wilkinson and Finar (12) first synthesized flufenamic acid by reacting o-iodobenzoic acid with m-trifluoromethylaniline in potassium carbonate aqueous solution, in the presence of copper bronze. The crude product was purified via its ammonium salt.

Moffett and Aspergren (19) prepared flufenamic acid starting from o-chlorobenzoic acid which was reacted with m-trifluoromethylaniline in 85% aqueous potassium hydroxide and amyl alcohol with copper powder. Some patented synthetic methods follow the latter scheme, as illustrated in Figure 5.

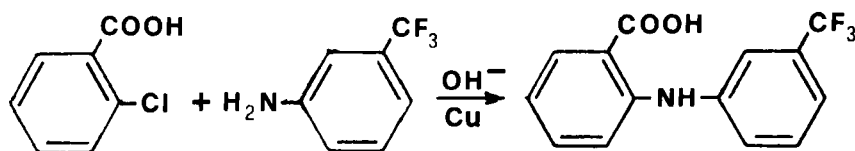


Figure 5
Synthesis of Flufenamic Acid

Flufenamic acid was also obtained via the reaction between o-iodobenzoic acid and m-trifluoromethylphenylhydroxylamine (34). Another method involves the reaction of

methyl o-chlorobenzoate with N-(3-trifluoromethylphenyl)-formamide (35). Flufenamic acid was also prepared by photolysis of the corresponding benzotriazinone (36).

4. DRUG METABOLISM AND PHARMACOKINETICS

4.1. Metabolism

The metabolic transformations which flufenamic acid undergoes in man and animals are depicted in Figure 6, according to Glazko (37) and Ober et al. (38), which demonstrated that the drug is excreted mainly in form of its metabolites. Their studies were carried out by tracer methods using [^{14}C]-carboxyl-labelled flufenamic acid. The 4'-hydroxy- and 5-hydroxy-derivative and flufenamic acid itself are eliminated in urine chiefly in conjugated form, whereas the 4',5-dihydroxy-derivative is not conjugated. All four compounds have been found in the stool in unconjugated form.

4'-Hydroxy- and 5-hydroxyflufenamic acids were synthesized by Bowman et al. (39).

4.2. Absorption and Excretion

4.2.1. In Animals

The rate of permeation of flufenamic acid through the gut membrane and the amount of drug absorbed were studied in vitro on segments of the small intestine of golden hamsters (20). The permeation of flufenamic acid was pH-dependent, according to the postulation that it is only the unionized molecule of the drug that passes through a cell, due to its lipid solubility. This study showed that at pH 2.5 the permeation of flufenamic acid, which is approximately 96% unionized, is 20 times faster than at pH 7.2, at which only 0.05% of the drug is in the unionized form.

The absorption and the urinary excretion of flufenamic acid was studied in rabbits following both cutaneous and oral application (40) of the same dose (30 mg/kg). After 48 hours from cutaneous application, 5.9% of the applied dose was found in the urine; the blood level of flufenamic acid remained constant over the first six hours at about 3 $\mu\text{g/ml}$, overcoming the blood level obtained orally as from the 4th hour.

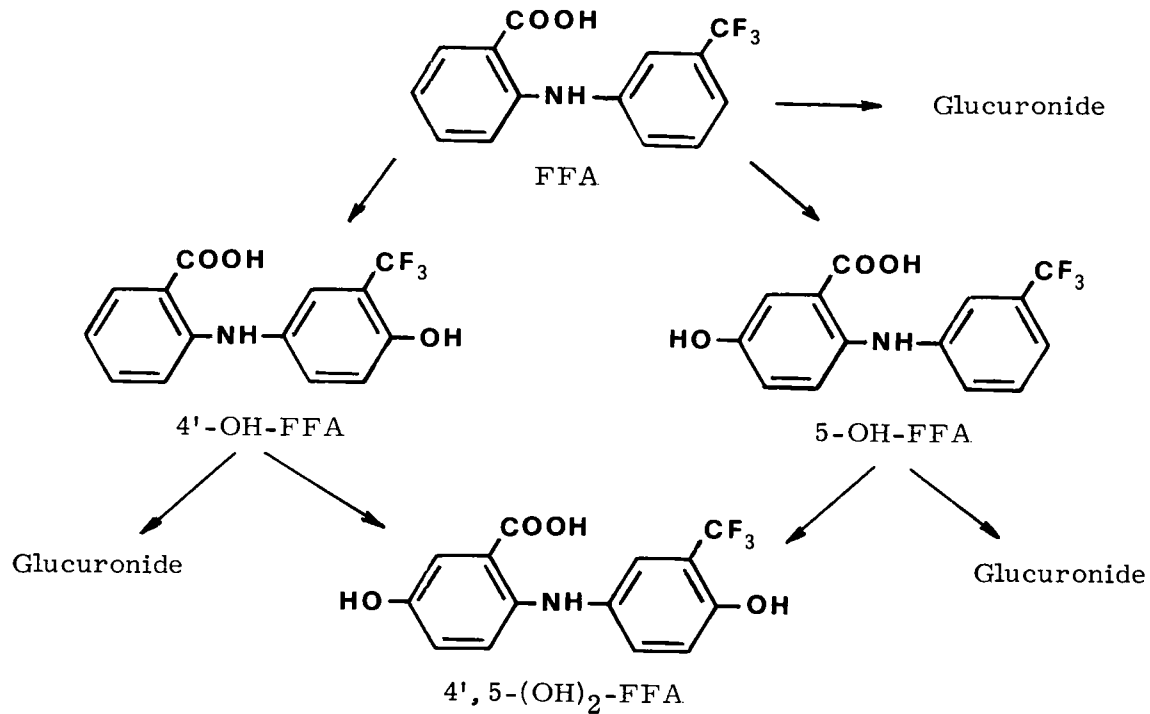


Figure 6
Metabolism of Flufenamic Acid (FFA)

Rosenberg and Bates (41) compared the blood concentrations produced in rats following oral administration of flufenamic acid alone or together with cholestyramine. Flufenamic acid strongly bound to the resin, so that a 60-70% decrease in both the rate and the extent of absorption of the drug was observed: following an oral dose of 50 mg/kg a peak plasma concentration of 89.2 $\mu\text{g/ml}$ was reached at 1 hour, whereas when cholestyramine and the drug were coadministered the peak level dropped to 32.3 $\mu\text{g/ml}$. Further studies on pharmacokinetics of flufenamic acid in the rat are reported. Frey and El-Sayed (30) determined the flufenamic acid concentrations in serum and gastric mucosa after oral and subcutaneous administration. Lin et al. (42) determined plasma levels after intravenous administration. Cotellessa et al. (43) determined plasma and uterus levels after intravenous and oral administration.

As regards the excretion, Glazko (37) showed that dogs eliminated only 2-7% of an oral dose in the urine and 53-79% in the feces, whereas the corresponding values for monkeys were 45-80% and 12-21%, respectively. Similar results were obtained by Ober et al. (38) in experiments with dogs. Lombardino et al. (25) reported that the serum half life after oral administration of the aluminium salt of flufenamic acid was 3 hours for rats and dogs, and 4 hours for rabbits.

4.22. In Humans

In addition to the results obtained in rabbits (40), Panse et al. have also studied the absorption of flufenamic acid through the human skin (44).

Glazko (37) reported that nearly 100% of an oral dose of flufenamic acid was absorbed; the renal elimination of the drug and its metabolites was 51%, of which only 2.6% was unaltered drug. Such results were more recently confirmed by Dell et al. (45,46) with two different methods for the determination of all fluorine-containing compounds as a group in the urine, which showed that the renal elimination of flufenamic acid and its metabolites was 49.4% within three days after oral administration. The peak plasma level was reached after two hours, and the plasma elimination half life was found to be approximate-

ly 3 hours. Dell et al. (45) also reported that 3.6% of an oral dose of flufenamic acid was excreted unconjugated into the urine within 6 days: however, female subjects eliminated only 1.9% and the male ones 5.3%. On the contrary, no difference was observed between men and women in the total amount of all metabolites excreted by the renal route. Another study on the sex-dependence of the renal excretion of flufenamic acid and other fenamates in man and animals was reported by Lorenz and Dell (47).

The bioavailability of oral pharmaceutical formulations of flufenamic acid was investigated by Arias and Cadorniga (48) and Angelucci et al. (49).

4.3. Protein binding

The bovine and human serum albumin (BSA and HSA, respectively) binding affinity of flufenamic acid was investigated by Chignell by circular dichroism studies (50-52). The role of hydrophobicity for the binding affinity was investigated by Dunn on HSA (23) and by Terada et al. on BSA (24). The hydrophobicity as well as the withdrawing ability of the $-\text{CF}_3$ substituent contribute significantly to the binding affinity, which was determined for BSA by measuring the ability of flufenamic acid to displace 2-(4'-hydroxyphenylazo)benzoic acid competitively under conditions of pH 7.0 and 25°C (24). The binding constant, K , was determined: the value obtained for BSA by Terada et al., $6.5 \times 10^5 \text{ l.mol}^{-1}$, seems to conform to a value, $1.3 \times 10^6 \text{ l.mol}^{-1}$, obtained by Chignell with HSA at pH 7.4 (51).

The interaction between BSA and several cationic and anionic drugs including flufenamic acid was studied by Blanchard et al. (53) using the electron spin resonance spin labeling technique.

The binding of flufenamic acid to HSA was studied by Otagiri et al. (54) via microcalorimetric investigations. The heat flux generated by the binding is proportional to the amount of the drug bound to the protein. If only one binding site on the drug molecule contributes to the heat flux, then the data can readily be interpreted in terms of the binding constant, ΔG , ΔH , and ΔS for binding to that site. If many sites are involved having different enthal-

pies of binding, then unambiguous interpretation of the data may be impossible. This is the case of flufenamic acid, which has been reported to have three very high affinity sites for HSA and other sites of lower affinity(51).

Sudlow et al. (55,56) have characterized two distinct binding sites (I and II) for anionic drugs on HSA by the use of fluorescent probes. Flufenamic acid binds selectively to site II, as well as ibuprofen, flurbiprofen and ethacrynic acid, whereas phenylbutazone and warfarin bind to site I. Drugs which bind to site II are all aromatic carboxylic acids, which would be largely ionized at physiological pH.

Kaneo et al. (57) examined the binding to BSA of six nonsteroidal antiinflammatory drugs including flufenamic acid by the use of dialysis at pH 7.4 and 37°C. It was found that flufenamic acid strongly binds to BSA and the free fraction of this drug exists within 1% over the therapeutic range.

5. METHODS OF ANALYSIS

5.1. Identification Tests

Flufenamic acid can be identified by virtue of its UV, IR, NMR, mass and fluorescence spectra (see Section 2). Various chromatographic methods are also suitable for purposes of identification (see Section 5.7).

Devaux et al. (58) described two color reactions and one fluorescent reaction. The color reactions are due to the diphenylamine structure, whereas the fluorescent reaction was explained by the formation of substituted acridones (see Section 5.5). These reactions can be carried out as follows:

a) Flufenamic acid (at least 1 mg) and about 0.5 g of oxalic acid are heated into an oil bath at 180-200°C 4-5 minutes. After cooling the residue is dissolved in 95% ethanol to obtain a stable, intense blue color. The absorption maximum is at 585-590 nm.

b) Flufenamic acid (at least 100 μ g) is added in a mixture of 1 ml $\text{CH}_3\text{COOH}:\text{H}_2\text{SO}_4$ (d.1.83)(98:2), 5 ml $\text{CH}_3\text{COOH}:\text{HCl}$ (d. 1.18)(50:50), and 1 ml 0.10% aqueous levulose. The mixture is heated at 100°C 25 minutes to ob-

tain a violet color. The absorption maximum is at 597 nm.

c) Flufenamic acid is dissolved in conc. H_2SO_4 and heated 10 minutes at 100°C : the solution exhibits an intense green fluorescence when excited by white light, and blue when excited by UV light.

5.2. Titrimetric Analysis

Flufenamic acid can be titrated in acetone with 0.1 N aqueous potassium hydroxide in the presence of phenolphthaleine (3). A nonaqueous titrimetric method was described by Walash and Rizk (59), which used 0.1 N sodium methoxide as the titrant and DMF or tetramethylurea as the solvent, measuring the end point either with a thymol blue indicator or potentiometrically. Various potentiometric methods in aqueous solvent systems are cited in Section 2.9.

5.3. Colorimetric Analysis

Flufenamic acid and its metabolites were determined as a group colorimetrically in urine after alkaline hydrolysis and fusion with sodium peroxide: fluorine was then distilled as H_2SiF_6 in the presence of H_2SO_4 and SiO_2 . A solution of alizarine-3-methylamino-N,N-diacetato-cerium(III) was added to the distillate to obtain a color reaction, and a colorimetric determination was effected at 617 nm. Measurements in the range $\leq 1 \mu\text{g}$ are possible (46).

The color reactions cited in Section 5.1 can also be used for quantitation.

5.4. Spectrophotometric Analysis

The first UV spectrophotometric method for fenamates analysis was described by Carey (60). The UV absorbance of flufenamic acid (see Section 2.3) either in methanol at 288 nm (3, 40, 44) or in 0.1 N NaOH at 287-290 nm (4, 30, 41) can be used for quantitative analysis. Beltagy (61) described a spectrophotometric method for the determination of several acidic drugs including flufenamic acid which was determined obtaining the ion-pair association complex of the drug and safranin in a pH 7.4 buffer, then extracting the complex with chloroform and measuring the absorbance of the extract.

5.5. Fluorometric Analysis

Mehta and Schulman (9) affirmed that the native fluorescence exhibited by flufenamic acid in organic solvents (see Section 2.5) could be useful for its detection and determination. Nevertheless the method most commonly used involves the fluorometric determination of flufenamic acid in carbon tetrachloride after addition of a CCl_4 solution of trichloroacetic acid which strongly increases the fluorescence intensity (11). The fluorescence maxima under this condition are reported in Section 2.5. This method was used by a number of authors to assay flufenamic acid in body fluids and tissues (8, 37, 40, 49, 62, 63, 64).

The reaction of flufenamic acid with formaldehyde gives 1-(m-trifluoromethylphenyl)-4-oxo-1,2-dihydro-3,1-benzoxazine (4), which is suitable for fluorometric determination of the parent drug (63). The reaction scheme is depicted in Figure 7. The methanolic solution of the benzoxazine derivative shows two excitation maxima at 278 and

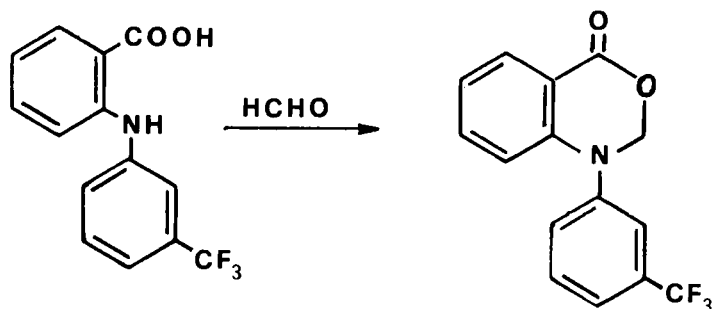
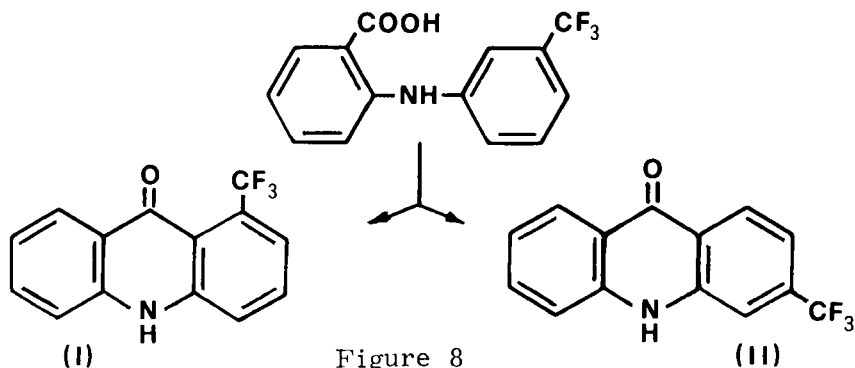


Figure 7

Reaction of Flufenamic Acid with Formaldehyde

342 nm and an emission maximum at 440-450 nm (4). The corresponding values reported by Dell et al. (63) are 346 and 458 nm.

Another fluorogenic reaction of flufenamic acid, already cited in Section 5.1, was studied by Dell and Kamp (4). Flufenamic acid was heated with concentrated sulfuric acid to give a mixture of two isomeric acridones, I and II, as illustrated in Figure 8. The fluorescence features of these compounds, which had been previously synthesized by Wilkinson and Finar (12), are very similar so that



Reaction of Flufenamic Acid with conc. H_2SO_4

the quantitative determination can be made on the mixture. In Table XI are listed the wavelengths of excitation and emission maxima in neutral, acidic and alkaline methanol (4).

TABLE XI

Fluorescence Data of Trifluoromethylacridones

Solvent system	Excitation/emission maxima, nm, of:	
	4- CF_3 -acridone (I)	2- CF_3 -acridone (II)
Methanol	400/420	400/421
Methanol-HCl	400/440	400/440
Methanol-NaOH	400/455-462	400/460

Hattori et al. (65) proposed a fluorometric method which involves the treatment of an ethanolic solution of flufenamic acid with 0.5% AlCl_3 solution in absolute ethanol to obtain an aluminium chelate, which fluoresces at 440 nm following activation at 358 nm. The maximum fluorometric sensitivity of flufenamic acid claimed for this method is 4 ng/ml.

5.6. Indirect Atomic Absorption Analysis

It was found that flufenamic acid, copper and 2-(2-hydroxyethyl)pyridine combined in the ratio 1:1:1 to form a chelate complex (18). To obtain this result the sample containing the drug was treated with a reagent prepared adding 9.0 ml of 0.1% cupric sulfate solution to

1.5 ml of 2-(2-hydroxyethyl)pyridine. The complex was then extracted with propyl acetate, and the amount of flufenamic acid present in the sample was obtained indirectly from the amount of copper determined in the organic solvent by atomic absorption analysis.

5.7. Chromatographic Analysis

5.71. Paper Chromatography

Flufenamic acid can be detected by paper chromatography (66) using the following solvent systems:

a) Methyl isobutyl ketone : formic acid : water (10 parts of ketone saturated with 1 part of 4% formic acid); $R_f = 0.95$.

b) Chloroform : methanol : formic acid : water (a mixture of 1 part of methanol and 1 part of 4% formic acid used to saturate 10 parts of chloroform); $R_f = 0.95$.

c) Benzene : methyl ethyl ketone : formic acid : water (a mixture of 9 parts of benzene and 1 part of ketone saturated with 1 part of 2% formic acid); $R_f = 0.94$.

d) Benzene : formic acid : water (10 parts of benzene saturated with 1 part of 2% formic acid); $R_f = 0.91$.

e) Methyl ethyl ketone : diethylamine : water (921:2:77); $R_f = 0.83$.

f) Methyl ethyl ketone : acetone : formic acid : water (40:2:1:6); $R_f = 0.95$.

Flufenamic acid can be visualized by UV light, or spraying the paper with 0.4% p-nitrobenzenediazonium fluoroborate solution in 1:2 dioxane:water or with 2% aqueous phosphomolybdic acid solution.

Schmollack and Wenzel (67) developed a method for the detection and quantitative determination of flufenamic acid using a chamber paper analysis apparatus. Flufenamic acid was then determined fluorometrically after treatment with formaldehyde vapor to obtain the strongly fluorescent benzoxazine derivative.

5.72. Thin Layer Chromatography

Several thin layer chromatographic methods have been developed for identification and quantitative determination of flufenamic acid. Some details of these methods are summarized in Table XII. It has to point out that in all cases silica gel plates were used.

TABLE XII

Thin Layer Chromatography of Flufenamic Acid

Ref.	Solvent System	Plates	R _f	Detection
3	Cyclohexane:CHCl ₃ :CH ₃ OH:CH ₃ COOH (60:30:5:5)	S. G. GF	0.54	UV(254nm)
3	Benzene:ether:CH ₃ COOH:CH ₃ OH (120:60:18:1)	id.	0.78	id.
4	Benzene:C ₂ H ₅ OH:CH ₃ COOH (20:2:1)	S. G. HF	NR	UV following heating with HCHO
8	Cyclohexane:ethyl acetate:CH ₃ COOH (20:30:2)	S. G. F ₆₀	0.58	UV(356 nm)
8	Cyclohexane:CHCl ₃ :CH ₃ COOH (40:50:10)	S. G. HF	NR	id.
40	id.	S. G. HFF	0.43	UV
44	id.	S. G. HF	NR	UV
63	id.	id.	0.58	UV; heat. HCHO + UV; iodine
63	Benzene:methanol (9:1)	id.	0.23	id.
63	Cyclohexane:ethyl acetate (1:10)	id.	0.23	id.
41	Isopropanol:ammonia:water (20:1:2)	NR	0.64	NR
68	Toluene:acetic acid (9:1)	S. G. G	0.73	HNO ₂ spray
68	Toluene:acetic acid (97.5:2.5)	id.	0.60	id.
69	Chloroform:methanol(7:3) in NH ₃ atm.	S. G. 60	0.37	HCHO/HCOOH at 100°C + UV
S. G. : silica gel. NR : not reported.				

Unterhalt (3) has identified flufenamic acid in a mixture with other nonsteroidal antiinflammatory drugs: the spot visualized by UV light was eluted with methanol and flufenamic acid was determined spectrophotometrically at 288 nm with a mean recovery of 88%.

Dell and Kamp (4) assayed flufenamic acid in urine and serum: their method involved an ethereal extraction from the biological fluids followed by TLC. The plates were then exposed to formaldehyde vapor (2 hours at 80°C) and the UV-fluorescent spot was eluted with methanol and flufenamic acid determined fluorometrically. An alternative method proposed by the same authors involved the visualization of flufenamic acid by UV light and a treatment of the scraped off silica gel with conc. H_2SO_4 followed by fluorometric determination.

The method described by Panse et al. (40) for the determination of flufenamic acid in urine and plasma was similar, involving extraction from biological material, TLC and elution of the drug, followed by quantitative determination either spectrophotometrically in methanolic solution or fluorometrically in a CCl_4 solution in the presence of trichloroacetic acid. The latter method was applied by several authors (already cited in Section 5.5) for the determination of flufenamic acid in biological fluids and tissues. Panse et al. (44) described also a method for the direct densitometric determination of flufenamic acid on the silica gel thin layers.

Flufenamic acid was determined directly in plasma by Geissler et al. (69) by addition of methanol to precipitate proteins, TLC, treatment of the dried plate with formaldehyde vapor in the presence of formic acid at 100°C for 45 minutes to form the benzoxazine derivative, and direct fluorimetry of the plate. Use of formic acid shortens the reaction time and enhances the fluorescence intensity and the sensitivity (quantities ≤ 2 ng/spot may be detected).

A method involving reversed-phase thin layer chromatography was reported by Boltze and Kreisfeld (2), which used silica gel plates impregnated with a 10% solution of Dow-Corning 200 in ether. Flufenamic acid was chromatographed using buffer:dioxane:acetone (2:1:1) as the solvent system, in which the buffer was at pH 5.2, 6.2, and

7.2, respectively. In Table XIII are listed R_f and R_M values found for flufenamic acid.

TABLE XIII
Reversed-phase TLC of Flufenamic Acid

<u>pH of the buffer</u>	<u>R_f</u>	<u>$R_M = \log(1/R_f - 1)$</u>
5.2	0.78	- 0.55
6.2	0.76	- 0.5
7.2	0.69	- 0.350

5.73. Gas Chromatography

Roseboom and Hulshoff (70) have developed a rapid and simple clean-up and derivatization procedure that can be generally applied to the gas chromatographic determination of acidic drugs including flufenamic acid in plasma samples. The drug was extracted from acidified plasma with chloroform:isopropanol (95:5), which was then evaporated. The residue was dissolved in toluene, then the drug was back-extracted with a small volume of a methanolic 20% tetramethylammonium hydroxide solution (TMAH). The solution obtained was added to N,N-dimethylacetamide. After treatment with n-butyl iodide the drug was chromatographed as its n-butyl ester. A gas chromatograph equipped with a flame ionization detector was used. The glass columns (150 cm x 2 mm I.D.) were packed with 3% OV-1, 3% OV-17 or 3% SP-1000, all on 100-120 mesh Chromosorb W HP. The carrier gas (nitrogen) flow-rate was maintained at 20 ml/min. The recovery of flufenamic acid in the first extraction step with chloroform:isopropanol was 69%, but with toluene, which can also be used for the extraction from plasma, a recovery of 95% was achieved. Toluene has the advantage that no evaporation of the extract is necessary, and it can be extracted directly with the TMAH solution. In the back-extraction with TMAH a recovery of 62% was obtained. The retention times of the n-butyl ester of flufenamic acid with various stationary phases are listed in Table XIV.

TABLE XIV

Gas Chromatographic Data for Flufenamic Acid (70)

Stationary phase	Column temperature, °C	Retention time, sec
3% OV-1	200	234
3% OV-17	210	271
3% SP-1000	230	218

Another gas chromatographic method for quantitative determination of flufenamic acid in plasma was reported by Cotellessa et al. (43). Flufenamic acid was extracted from plasma with benzene, after dilution of plasma sample with an equal volume of 0.25 M acetate buffer (pH 4.35). The benzene extract was evaporated to dryness under vacuum. The residue was dissolved in methanol and methylated with diazomethane. The same procedure was also applied to rat uterus homogenates. The methylated sample was dissolved in an acetone solution of the internal standard 3-chloro-6-aminobenzophenone. Gas chromatographic analysis was carried out using a gas chromatograph equipped with a flame ionization detector. The stationary phase was 3% OV-17 on Gas-Chrom Q (100-120 mesh) packed into a glass column (3 m x 2 mm I.D.). The column temperature was 230°C and the carrier gas (nitrogen) flow-rate was 40 ml/min. FID sensitivity was 1 µg/ml plasma and 5 µg/g uterus.

A mass spectrometer coupled with the gas chromatograph was employed to ascertain the identity of the methyl ester of flufenamic acid with the GC peak. A mass spectrum of the methyl ester is presented. The recoveries from plasma in the range 10-100 µg/ml and from uterus homogenates were 98.2% and 90%, respectively.

A gas chromatographic method for the detection of non-steroidal antiinflammatory drugs including flufenamic acid in urine collected from horses that had received these compounds orally has been developed by Hunt et al. (71). This procedure involves the isolation of the drugs from

urine by solvent extraction and on-column methylation of the carboxylic acid group.

5.74. High Performance Liquid Chromatography

A method for the separation and determination of some nonsteroidal antiinflammatory drugs including flufenamic acid was described by Dusci and Hackett (72). This procedure, which can be applied to serum samples of small volume (100 μ l), involved the extraction of the drugs with acetonitrile. The extract was taken to dryness at 50°C under a stream of nitrogen. The residue was redissolved in 100 μ l of the elution solvent (60% acetonitrile in 45 mM KH_2PO_4 adjusted to pH 3.0 with H_3PO_4). An aliquot of 10-20 μ l was injected in a high performance liquid chromatograph equipped with a variable wavelength UV detector. The column (30 cm x 3.9 mm I.D.) was packed with μ Bondapak C_{18} . The conditions for individual analysis of flufenamic acid were as follows: flow-rate of the elution solvent 2.0 ml/min, wavelength 282 nm. For the separation of flufenamic acid from the mixture of antiinflammatory drugs (flufenamic and mefenamic acid, naproxen, ibuprofen, indomethacin, phenylbutazone, oxyphenbutazone) a flow-rate of 0.8 ml/min and a wavelength of 225 nm was used: under these conditions the elution time of flufenamic acid was 10.5 min.

Using the above-mentioned elution solvent, flufenamic and mefenamic acid were not separated. A modified elution solvent (35% acetonitrile in 0.7% NH_4Cl buffered to pH 7.8 with ammonia) allowed to obtain the separation of all the drugs investigated. Using a flow-rate of 1.0 ml/min, the elution time of flufenamic acid was 10.2 min (mefenamic acid 7.8 min). The recovery of flufenamic acid was 92 \pm 3% in a series of ten plasma samples examined, in the range 1.0-20 μ g/ml.

Lin et al. (42) have developed a HPLC procedure for the determination of flufenamic acid and mefenamic acid in plasma. A single extraction step is followed by reversed-phase chromatography. Flufenamic acid and mefenamic acid can be internal standards for each other during either assay. The extraction of flufenamic acid from acidified plasma samples (1 ml), to which 4 μ g of mefenamic acid had been added, was accomplished with carbon tetra-

chloride. The extract was evaporated to dryness under a stream of nitrogen and the residue was redissolved into 0.5 ml of methanol, and an aliquot was injected in the chromatograph, which was equipped with a stainless steel column (30 cm x 4 mm I.D.) packed with a stable reversed-phase stationary phase of porous silica beads coated with chemically bonded cyanopropylsilane monolayers. The elution solvent was water:acetonitrile:acetic acid (60:30:10). The flow-rate was 1 ml/min with an operating pressure of 1000 psi at room temperature. The effluent was monitored continuously at 254 nm. Under these conditions flufenamic acid had an elution time of 10.4 min, with a mean recovery from plasma of $100.7 \pm 3.4\%$ in the 1-10 μg range. The sensitivity limit was 1 $\mu\text{g}/\text{ml}$ of plasma.

A method for the determination of flufenamic acid and its major metabolites, 4'-hydroxy- and 5-hydroxyflufenamic acid, was described by Kubo et al. (73): the acidified serum was extracted with ethyl acetate. The organic extract was evaporated and the residue was redissolved in ethanol and chromatographed, using a column packed with Bondapack C₁₈. The mobile phase consisted of water:ethanol (52:48) containing 0.1% Na₂HPO₄ and 0.5% tetrabutylammonium bromide, adjusted to pH 7.81. The recoveries from plasma were 98.8% for flufenamic acid, 97.0% for 4'-hydroxy-derivative, and 98.0% for 5-hydroxy-derivative.

6. DETERMINATION IN BODY FLUIDS AND TISSUES

Many methods among those outlined in this analytical profile have been applied to the detection and quantitative determination of flufenamic acid in biological samples of animal or human origin. Such applications were reported in the papers listed below :

Colorimetry	46
Spectrophotometry	4, 30, 40, 41, 44, 60, 61
Fluorimetry	4, 8, 11, 37, 40, 45, 47, 49, 62, 63, 64, 65, 67, 69
Indirect AA Analysis	18
PC	67
TLC	4, 8, 40, 41, 44, 45, 63, 64, 68, 69

GC	43, 70, 71
HPLC	42, 72, 73

7. ACKNOWLEDGEMENT

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HEXESTROL

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ANALYTICAL PROFILE-HEXESTROL

1. Description1.1 Nomenclature1.11 - Chemical Names

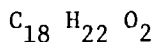
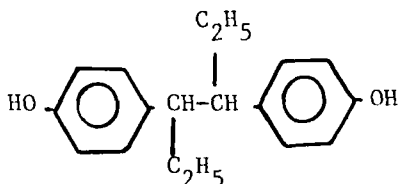
- (±) 3, 4 - Di(p-hydroxy phenyl) n-hexane.
 4, 4'- (1, 2 diethyl - 1, 2 - ethanediyl)
 bis-phenol.
 4, 4'- (1, 2 diethylethylene) diphenol.
 4, 4'- dihydroxy - γ,δ-diphenyl hexane.
 4, 4'- dihydroxy - α,β-diethyldiphenylethane.
 p, p'- dihydroxy - diphenyl hexane.
 meso - 3, 4 - bis (p-hydroxyphenyl) -n-
 hexane.

1.12 - Generic Name

Hexestrol, Hexoestrol, Dihydrodiethyl-
 stilbestrol, Hexanoestrol, Cycloestrol,
 Hormoestrol, Synestrol.

1.13 - Trade Name

Syntrogène, Folliplex, Synthovo.

1.2 Formula1.21 - Emprical1.22 - Structural1.3 Molecular Weight

270.4

1.4 Elemental Composition

C,79.96%; H,8.2%; O,11.84%

1.5 Appearance, Color, odor

white, odorless, colorless crystals or crystalline powder (1).

2. Physical properties2.1 Crystal properties

The following table shows the crystal form of hexestrol derivatives and their melting points. These derivatives can be used for identification purposes too (2).

Derivative	Crystallization solvent	Crystal form	M.P., °C
------------	-------------------------	--------------	----------

Meso Form

Di-Me ether	Me ₂ CO-MeOH	plates	146
Dipropionyl	Pet. ether	crystals	127-128
Dibutyryl	Pet. ether	crystals	106-107
Dicaproyl	Pet. ether	crystals	96-97
Disuccinyl	CHCl ₃ -pet. ether	crystals	150-153

DL(±) Form

Di-Me ether	C ₆ H ₆ -pet. ether	56
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2.2 Melting point

The following are the melting points for hexestrol in its meso, DL-forms and their antipodes.

Form	M.P., °C	Ref
------	----------	-----

Meso

185-188	3
184-185	4
186	5

DL(±) Form

128	2
-----	---

<u>L(-) Form</u>	80	2
<u>D(+)</u> Form	80	2

2.3 Solubility

Freely soluble in ether, soluble in acetone, alcohol, methanol, soluble in vegetable oils upon warming also soluble in dilute solution of alkali hydroxide. Slightly soluble in chloroform, benzene; insoluble in water (1, 3).

2.4 Identification

The following identification tests are described in the Pharmaceutical Codex 1979 (5).

- 1) To about 250mg add 1ml of acetic anhydride and 2ml of dehydrated pyridine, boil under a reflux condenser for 15 minutes, cool, add 50ml of water and shake thoroughly until a precipitate is produced which, after washing with water and drying, melts at about 138°C.
- 2) Dissolve about 10mg in 5 ml of H₂SO₄; the solution is colorless (distinction from stilboestrol, which gives a golden-yellow color).

2.5 Spectral properties

2.51 Ultraviolet spectra

Hexestrol in ethanol shows maxima at 230nm (E₁%, 1cm, 775) and 280 nm (E₁%, 1cm, 140); 0.1N NaOH hexestrol gives maxima at 242 nm (E₁%, 1cm, 965) and 295 nm (E₁%, 1cm, 175). As shown in figure (1) and in agreement to the figures published by Clarke (3).

2.52 Infrared Spectra

The infrared spectrum of hexestrol in KBr disc is given in figure (2). Major band assignments are as follows:

<u>Frequency Cm⁻¹</u>	<u>Assignment</u>
3400	Phenolic OH
1610, 1600	Aromatic ring C=C stretch

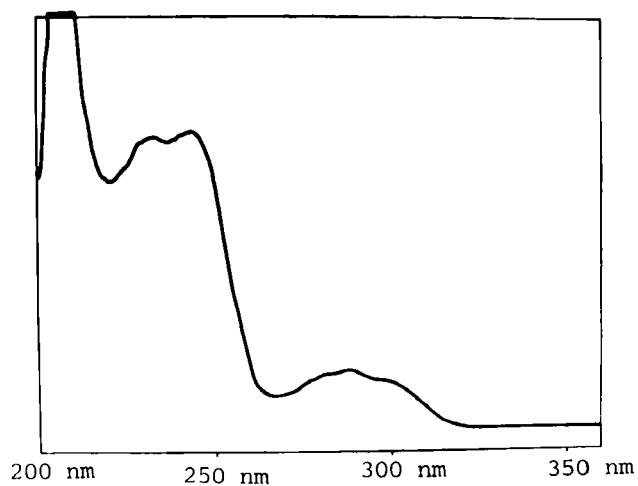


Fig. 1. The ultraviolet absorption spectrum of Hexestrol in ethanol.

Instrument: Pye Unicam SP8-100

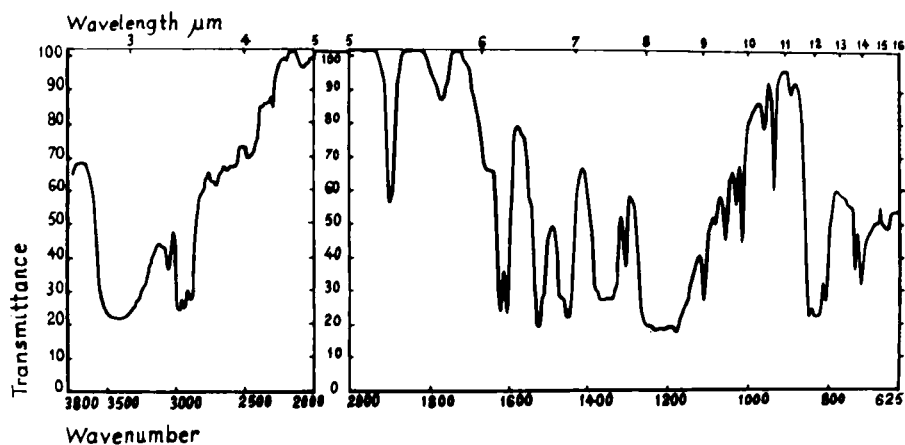


Figure 2. IR spectrum of Hexestrol in KBr.

Instrument: Perkin Elmer 567

Other finger print bands characteristic to hexestrol are 1530, 1450, 1300 and 1110 cm^{-1} .

2.53 Nuclear Magnetic Resonance Spectrum

a) PMR

A typical PMR spectrum of hexestrol is shown in Figure (3).

The sample was dissolved in deuterated CDCl_3 and a drop of deuterated dimethyl sulfoxide (DMSO-d_6) using TMS as the internal standard.

The spectrum was determined on a Varian T60-A spectrometer.

The following structural assignments have been elicited from Figure (3).

<u>Chemical Shift (δ)</u>	<u>Assignment</u>
Triplet at 0.50	CH_3CH_2-
Multiplet centered at 1.3	CH_3CH_2
Multiplet centered at 1.90	$-\text{CH}-\text{CH}-$
Doublet of doublet centered at 6.86	eight aromatic protons characteristic for para substitution of the ring.
Broad singlet exchangeable with D_2O at 7.67	phenolic OH group.

b) ^{13}C -NMR

The ^{13}C -NMR of hexestrol has been determined,* the off-resonance spectrum (Fig. 4) shows seven singlets. The complete spectrum is shown in Fig. 5. The spectrum was determined on a Varian FT-80A in DMSO-d_6 as solvent, tube diameter 10 mm, spectral width 5000 Hz

*H.Y. Aboul-Enein, unpublished data

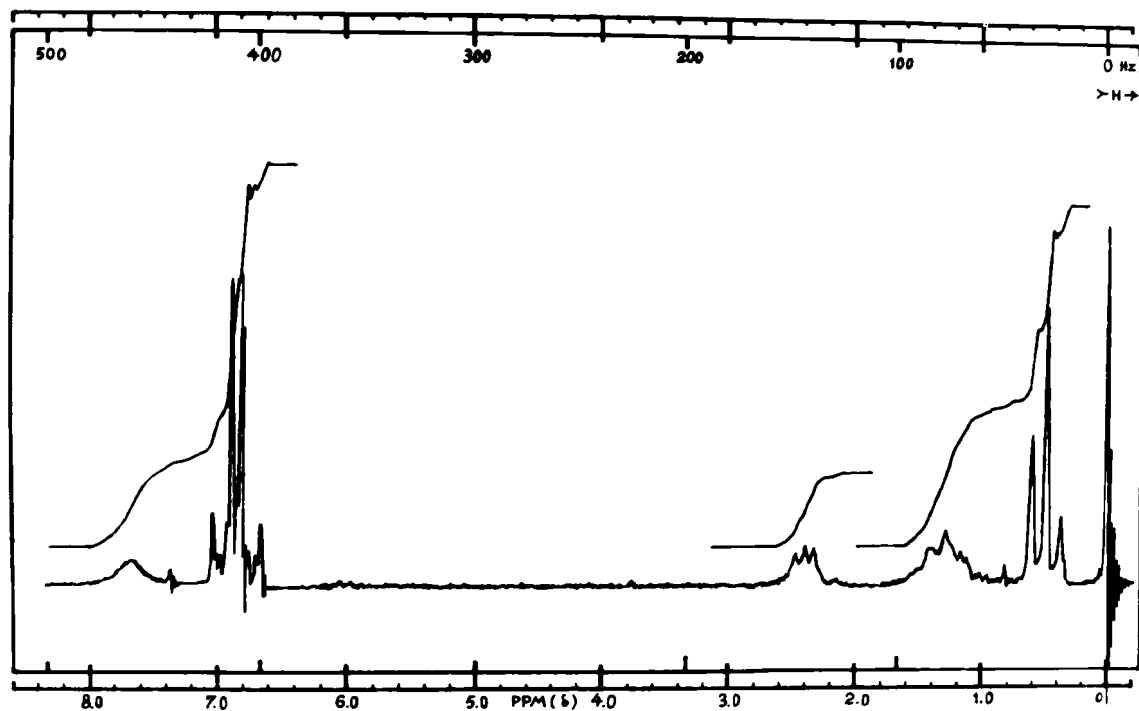


Figure 3. PMR spectrum of Hexestrol in CDCl_3 - DMSO-d_6 with TMS as internal standard.

Instrument: Varian T60-A

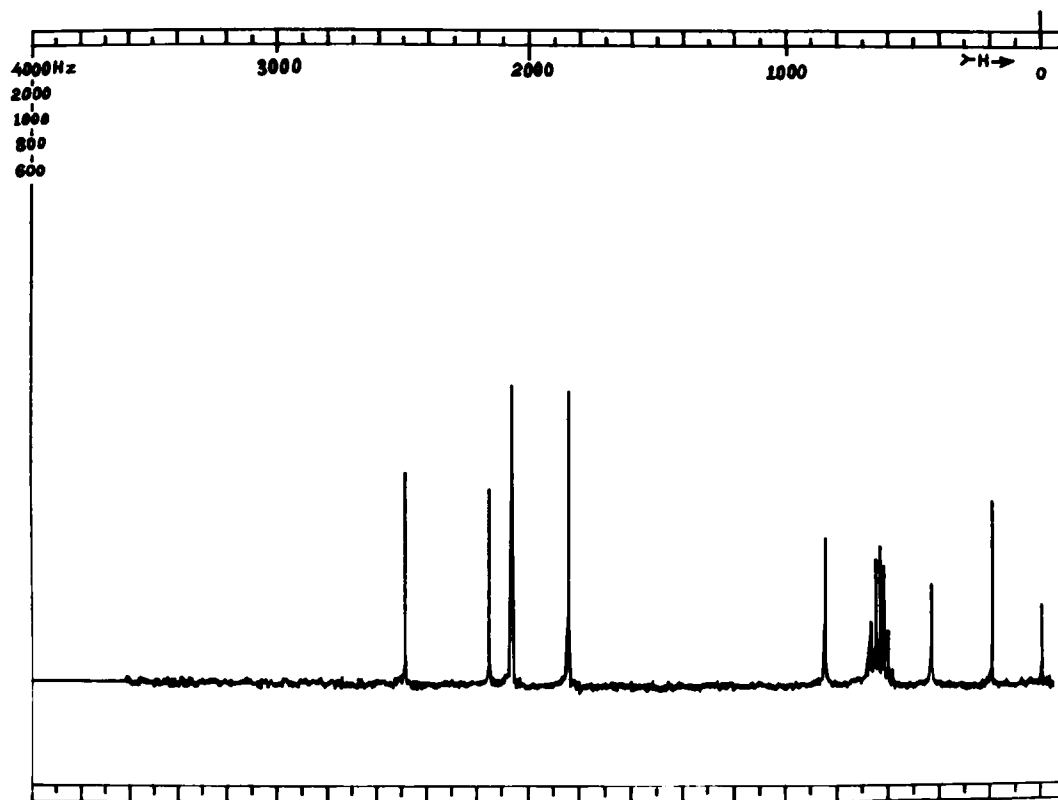


Figure 4. ^{13}C NMR off resonance spectrum of Hexestrol in DMSO-d_6

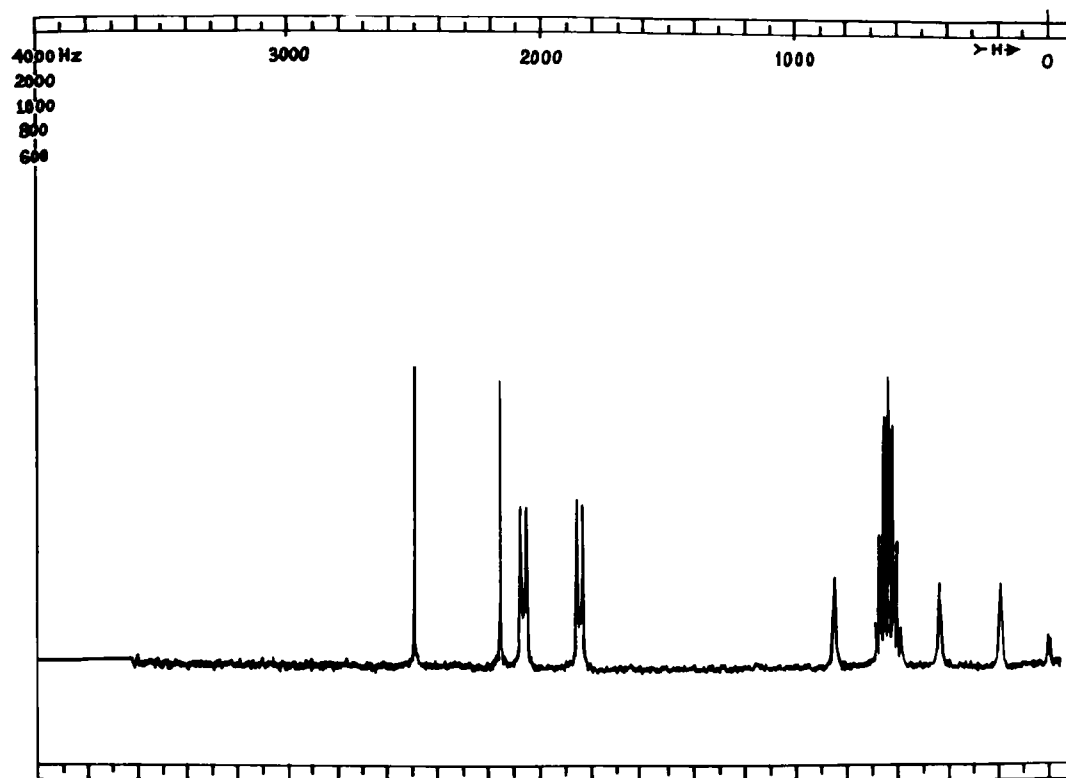
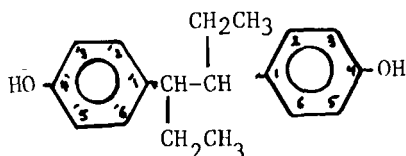


Figure 5. ^{13}C NMR proton-coupled spectrum of Hexestrol in DMSO-d_6

acquisition time : 1.638 sec.; pulse width :
4 μ sec; number of data print: 16384.

Spectral assignments are listed below:



Chemical Shift
(in ppm relative to TMS)

Assignments

12.06	$\underline{\text{CH}}_3$
26.97	$\underline{\text{CH}}_2$
52.77	$\underline{\text{CH}}-$
134.42	$\text{C}_1 - \text{C}_1$
115.05	$\text{C}_2 - \text{C}_6$ $\text{C}_2 - \text{C}_6$
128.82	$\text{C}_3 - \text{C}_5$ $\text{C}_3 - \text{C}_5$
155.39	$\text{C}_4 - \text{C}_4$

2.54 Mass Spectrum

The mass spectrum of hexestrol, obtained by chemical ionization with isobutane gas, is shown in Fig. 6. The spectrum was determined by direct inlet to Ribermag 10-10R mass spectrometer and exhibits comparatively little fragmentation.

The following table gives the most prominent ions and their relative intensities.

Mass (m/e)	Relative Intensity %
270	4.6 (M ⁺)
219	10.5
178	11.8
177	93
136	9.9

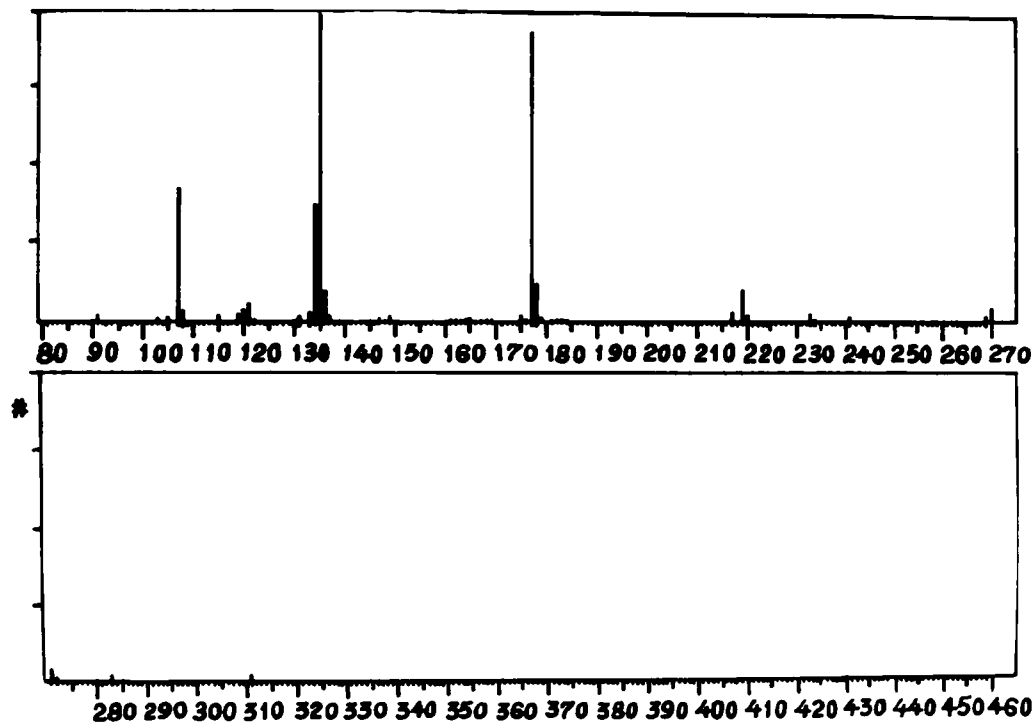
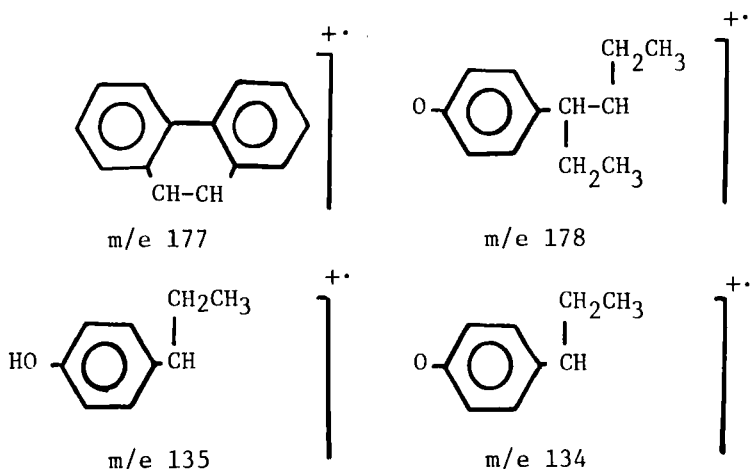


Figure 6. Mass Spectrum of Hexestrol (CI-isobutane) determined by direct inlet insertion.

135	100 (base peak)
134	36.9
107	42.6

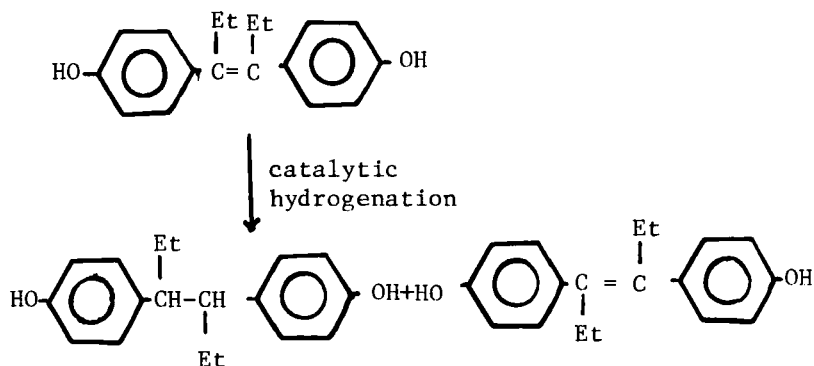


3. Synthesis

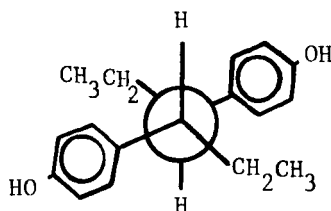
Several methods have been published and patented for the synthesis of hexestrol. In 1938 Campbell *et-al* (6) isolated hexestrol in poor yield, from the products of demethylation of anethole.

Some of the synthetic approaches for hexestrol are summarized as follows:-

- 1) Catalytic hydrogenation of pseudodiethylstilbestrol gives hexestrol along with some diethylstilbestrol (7).

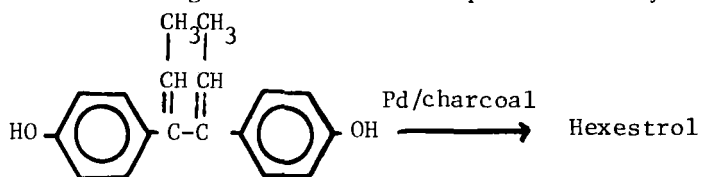


Hydrogenation of the dimethylethers of diethylstilbestrol and pseudodiethylstilbestrol with subsequent demethylation affords the meso-isomer of hexestrol which is more potent than the DL-isomer (7).

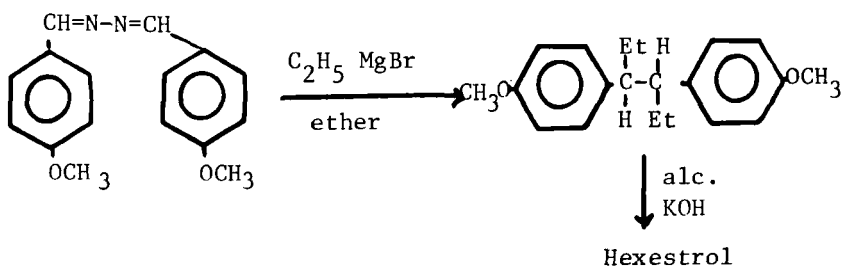


meso-form

- 2) Hydrogenation of 4,4' dihydroxy γ - δ diphenyl γ - δ hexadiene gives hexestrol in quantitative yield (7).

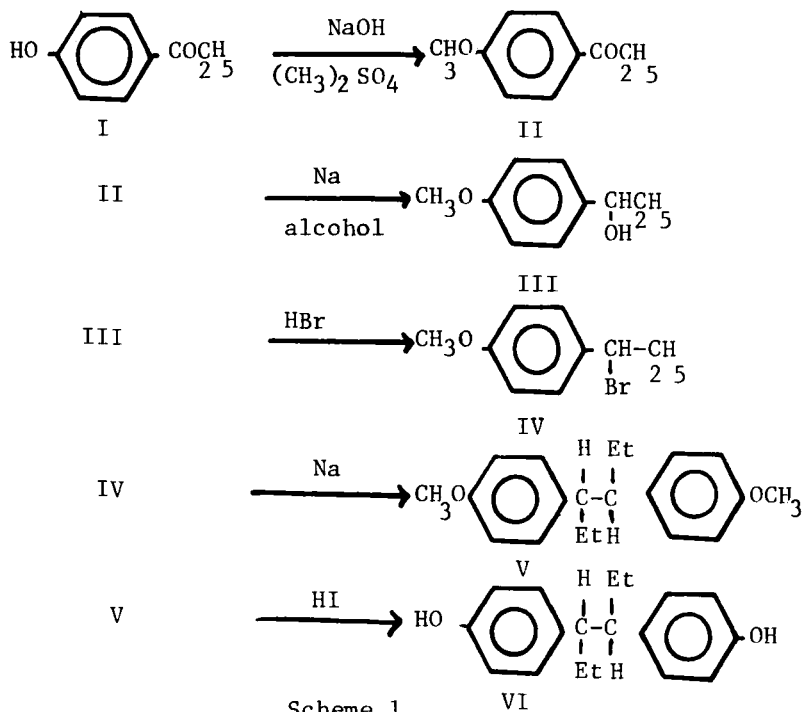


- 3) Hexestrol may be prepared by the action of ethyl magnesium bromide on anisaldazine with subsequent demethylation (8).

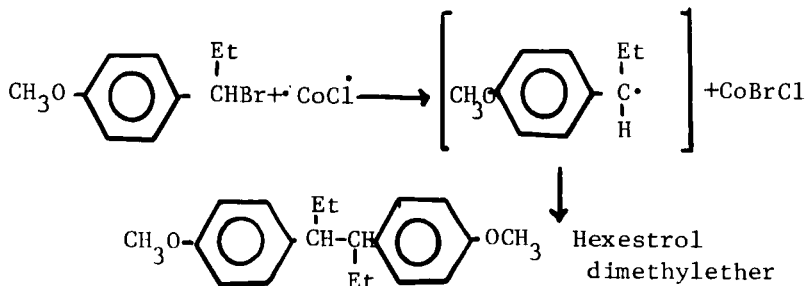


The above methods for the synthesis of hexestrol involve the use of intermediates which are difficult to prepare.

- 4) Bernstein and Wallis described (4) an alternate inexpensive method for the synthesis of hexestrol starting from p-hydroxypropiophenone as shown in scheme (1)



- 5) Kharasch and Kleiman(9) prepared hexestrol dimethylether from anethole hydrobromide and Grignard reagent in the presence of a halide of cobalt, nickel or iron, the free radicle generated from this reaction dimerizes to give hexestrol dimethylether in yields ranging from 14-41%. The higher melting point for the meso-form allows its separation from the DL-by-product formed in this reaction.

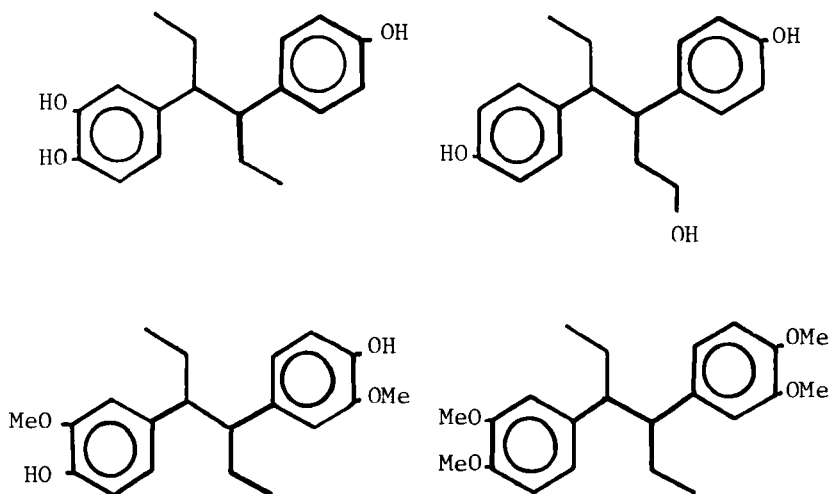


4. Stability and Decomposition products

Hexestrol is a relatively stable compound at room temperature; however it is recommended to be kept in a well closed container protected from light.

5. Metabolism

Hexestrol is metabolized in a similar way as diethylstilbestrol, it is excreted chiefly as a glucuronide conjugate (3). This glucuronide is mostly excreted into the bile which is subjected to hydrolysis by intestinal glucuronidase enzyme during its passage into the gut. This allows the drug be reabsorbed, re-conjugated and re-excreted (hepatic circulation) (10). Other possible metabolite intermediates which should be investigated are shown in scheme (2). This is in analogy to the possible metabolites of diethylstilbestrol which has recently attracted the attention by being linked to the occurrence of vaginal adenocarcinoma in adolescent daughters whose mothers had received diethylstilbestrol during pregnancy (11, 12, 13).



Scheme 2. Expected Metabolites of Hexestrol

6. Methods of Analysis

6.1 Titrimetry

- Aqueous (Bromometry)

The following method has been described for the quantitative determination of hexestrol diacetate (14): To 70 mg of sample add 10 ml of 0.5N methanolic KOH, and heat under reflux for 30 minutes on a water-bath. Cool, add 50ml of acetic anhydride, shake until the hydrolysate is dissolved, then add 2 ml of 30% KBr solution, 2 ml of conc. H_2SO_4 and 20 ml of 0.1N-KBrO₃, and set aside in the dark for 10 min. Add 1gm of KI and 100 ml of H_2O , and titrate with 0.1N-Na₂S₂O₃ using starch as indicator. The error is $\pm 1\%$ over the range 70 to 90 mg of hexestrol diacetate.

6.2 Colorimetry

Hexestrol has been determined in oily solution and tablet form as follows (15):

For tablet: extract a sample containing 2 to 3 mg of hexestrol with methanol, dilute the extract with water to 100 ml, and filter. To a 20 ml aliquot add borate buffer solution (pH 11).

For oily injections: Mix a sample containing 0.4 to 0.6 mg of hexestrol with light petroleum (5ml), shake the solution with methanol (6ml), add buffer solution (13ml), shake, and collect the aq. methanol phase in a 100ml flash. Twice repeat the extraction with methanol (6ml) and buffer solution (13ml) and to the combined aq. extracts add methanol (2ml).

For determination of hexestrol: heat the prepared solution on a water bath for 30 min., cool it to room temperature, add diazotized sulphanilic acid solution (12ml) and mix. After 40 minutes dilute with buffer solution to 100 ml, and measure the extinction at 495 nm against water. Beer's law is obeyed for 0.5 to 4 mg of hexestrol per 100ml. Results agree to within $\pm 10\%$ with theoretical values.

Belikov, described a similar procedure (16) which depends on diazodization of hexestrol with diazo-sulphanilic acid. However, the azo-dye formed is measured in alkaline medium at 420 nm.

Another colorimetric method for determination of hexestrol in feeds is reported as follows (17):

The ground feeding stuff (40gm) mixed with 10gm of sand is set aside with chloroform over night, then extracted with CHCl_3 for 6 hours. The extract is made up to 200ml of CHCl_3 . The residue from evaporation of the final CHCl_3 extract is dissolved in triethylamine - tetrahydrofuran-water (1:5:14), the solution is retained in an oiled cellulose column for 1 hour, then the impurities are eluted with triethylamine - tetrahydrofuran - water mixture. The column is acidified with $\text{N-H}_2\text{SO}_4$ and extracted with ethyl ether, the extract is evaporated, the residue is dissolved in ethanol, and to this solution are added water, dil. HCl , a molybdotungstophosphate reagent and then Na_2CO_3 solution.

The extinction of the centrifuged solution is measured at 750 nm against a reagent blank and compared with that of standard solution of hexestrol treated similarly.

6.3 Ultraviolet Spectrophotometry (U.V.)

Hexestrol was quantitatively determined in tablet spectrophotometrically (18). The drug was extracted with CHCl_3 from an acidified powdered sample (containing about 5mg of hexestrol). The CHCl_3 extract was concentrated and 2, 2, 4 trimethylpentane was added and hexestrol was extracted with 0.1N NaOH . The combined alkaline solution was acidified and re-extracted with CHCl_3 . The CHCl_3 solution was washed with water and dried over Na_2SO_4 and evaporated to dryness.

The residue was dissolved in ethanol and measured at 280nm. The percentage recovery ranged from 94.4-98.9%.

Kovalenko reported a quantitative method for the assay of hexestrol (19). About 25mg of hexestrol was weighed and dissolved in 25-30ml of 0.1N NaOH in a 50ml volumetric flask. The volume was adjusted to 50ml with 0.1N NaOH solution. Then pipette 5ml of this solution into a second 50ml volumetric flask and the volume adjusted

to 50ml with 0.1N NaOH solution. The same serial dilution was repeated and the concentration of hexestrol in the last solution was determined by measuring the absorption at 241 nm using 0.1N NaOH solution for the blank. Since these solutions follow the Lambert-Beer law $1.8 \mu\text{g ml}$, the determination can be made from a standard curve.

6.4 Chromatographic Analysis

6.41 Paper Chromatography

Tompsett has described a method for detection of hexestrol and other stilbestrol derivatives along with the p-hydroxymetabolites of phenobarbitone and phenytoin in urine using the 2-dimensional paper chromatography (20,21).

The two systems used were isopropanol: $\text{NH}_3(0.99):\text{H}_2\text{O}$ (8:1:1) and $\text{C}_6\text{H}_6:\text{EtOAc}:\text{H}_2\text{O}(2:1:1)$.

The detecting agents used were Pauly's reagent (red-brown), diazotized p-nitro-aniline (brown), diazotized diethylaminoethyl p-aminophenyl sulphone (brown) and 1-nitroso-2-naphthol nitric acid mixture (+ve). The sensitivity range for this method is 5-80 μg .

6.42 Column Chromatography

Column chromatography has been applied to purify the cattle feed extracts containing hexestrol and other stilbestrols to remove interfering substances before its determination. An example of the columns used for the purification of the extracts is Al_2O_3 column (22).

Verbeke (23) used columns containing XAD-2, Celite, or neutral Al_2O_3 (Brockman Activity I) for purification of tissue extracts and urine using distilled water; 15 ml water-washed ether followed by, 10ml C_6H_6 then benzene-isooctane(1:1); and benzene:isooctane (1:1) as eluents respectively for detection of hexestrol and other anabolics.

6.43 Thin Layer Chromatography

Several reports have been published on the detection, quantitative determination of hexestrol and other chemically related drugs for example diethylstilbestrol in feeds (meat, milk) and in biological fluids.

Table (1) summerizes the solvent systems and the detecting agents used in the cited references.

6.44 Gas-Liquid Chromatography

Various gas-liquid chromatographic methods have been developed for detection of hexestrol in meat and other agricultural products. Gain and Scholl (31) described a method for determination of hexestrol in molasses - based liquid feed supplements, after the preparation of the bis (trimethylsilyl) acetamide derivative. However the method showed interfering peaks or low recovery due to emulsion formation. Most of the gas chromatographic determination requires derivatization of hexestrol before injecting into the gas chromatograph.

Table (2) summerizes the data obtained from the litratures till 1980.

Table 1. (contd.)

Solvent system	Stationary phase	Detecting agent	Remarks	Ref.
2 dimensional TLC		5% H_2SO_4 -	sensitivity 0.5-	23
a) $\text{CHCl}_3:\text{EtOH}:\text{C}_6\text{H}_6$ (36:1:4)		induced fluore	10ppb	
b) $\text{n-C}_6\text{H}_{14}:\text{Et}_2\text{O}:\text{CH}_2\text{Cl}_2$ (4:3:2)		scence at 366nm		
petroleum ether (40-65°C):	Silica gel (activated at 120 oC reagent)	vanillin	applied to animal feeding stuff	27
2 dimensional TLC using:	Silica gel H			28
a) $\text{CHCl}_3:\text{EtOH}$ (9:1)				
b) $\text{C}_6\text{H}_6:\text{EtOAc}$ (3:1)				
<u>t.l.c</u>				
$\text{CH}_2\text{Cl}_2:\text{Me}_2\text{CO}$ (4:1)	Kieselgel or Kieselgel F ₂₅₄	ultraviolet at 254nm	sensitivity range 0.2.2 μg (the high performance tlc range 10-200ng	29

Table 1.

Solvent system	Stationary phase	Detecting agent	Remarks	Ref.
Toluene:EtOAc(19:1)	Aluminium oxide	Ultraviolet at 254 nm	Sensitivity 5-10ppb	24
C ₆ H ₆ :EtOAc(20:1)			(in fresh liver and kidney)	
CH ₂ Cl ₂ :Me ₂ CO(4:1)	Silica gel	Ultraviolet at 254 nm	recommended for routine test of estrogens in food	25
CHCl ₃ :EtOAc(4:1)				
2 dimensional TLC with				
a) n C ₆ H ₁₄ :Et ₂ O:CH ₂ Cl ₂ (4:3:2) in both direction or				
b) EtOAc:C ₆ H ₆ (1:3) after solvent system (a)				
n-C ₆ H ₁₄ :Et ₂ O:CH ₂ Cl ₂ (4:3:2)	Silica gel G	ultraviolet 254nm por 20'	sensitivity of the test is 0.5µg	26

Table 1. (contd.)

Solvent system	Stationary phase	Detecting agent	Remarks	Ref.
<u>h.p.t.l.c.</u>				
$\text{CH}_2\text{Cl}_2:\text{Me}_2\text{CO}(4:1)$	Kieselgel F ₂₅₄	densitometrically at 287 nm	(t.l.c. range 200-2000ng). (h.p.t.l.c range 10-20ng)	
$\text{C}_6\text{H}_{14}:\text{Me}_2\text{CO}(3:2)$ or $\text{C}_6\text{H}_6:\text{Me}_2\text{CO}(3:1)$	Polyamide		the influences of chemical structure and migration rate are discussed.	30

Table 2.

Column	Carrier Gas	Detector	Remarks	Ref.
3% OV-1 on Chromosorb WHP (80 to 100 mesh) at various temperature	Ar-CH ₄ (19:1)	electron capture	derivatized by heptafluorobutyric anhydride	32
	He	electrolytic conductor	sensitivity for electron capture (40-400 picog); and for electrolytic conductor (1-5 ng)	32
2% OV-17, 3%OV-1,3%OV-1 5% of neopentyl glycol sebacate or 2% Carbowax 20M on silanised Chromosorb W (80 to 100 mest) at various temperatures.	N ₂	electron capture	derivatized by hepta- fluorobutyric anhydride; sensitivity range 6 to 262 ng/ml	33
3% QF-1 on 100 to 200 mesh Gas. Chrom Q at 170°.		flame ioniza- tion detector	injected as a dipropionate derivative; sensitivity 0.05 mg/ml	34
Apiezon L-Epikote 1001(5:1) on silanised Chromosorb G at 160°C.	N ₂	Electron- capture	derivatized by trifluoroacetic anhydriode.	

6.45 High Performance Liquid Chromatography

Hexestrol has been determined among other anabolic synthetic and natural hormones in meat by HPLC. The columns used were Rp-2 (10 μm), Rp-18 (5 μm) or Zorbax CN (5 μm) by gradient elution with acetonitrile: H_2O (1:9) plus 25%, increasing rectilinearly in 5 minute to 45%, of acetonitrile: H_2O (9:1). The flow rate was 2ml min^{-1} , and 100 mg each of LiCl and LiClO_4 were added to each 100ml of eluent.

The eluate was examined by voltammetry with vitreous carbon electrodes and on silver-AgCl (3M-KCl) reference electrode; the potential sweep rate was 5mVs^{-1} .

Other columns used for the determination of hexestrol and other related drugs is Lichrosorb RP-8 with mixtures (35:13, to 25:23) of methanol and water (containing in all instances 2 parts of acetonitrile) as a mobile phase (0.8 to 1.6 ml min^{-1}). The voltammetric curve of the eluate was recorded by the use of a vitreous-carbon electrode, a platinum counter-electrode and a silver-AgCl reference electrode at pH 3, the peak potential(V) for hexestrol was +.9. Amounts range from 1-4 ng g^{-1} of hexestrol in meat could be determined by HPLC.

6.5 Mass Fragmentography

Hexestrol among other stilbestrol was quantitatively determined by combined GC/MS (38).

The sensitivity range is 40 part per 10^9 , the method is successfully used for detection of estrogens and in meat products. It involves extraction of liver, kidney and muscle tissue in acetonitrile: water (9:1). The drug was converted to its trimethylsilyl derivative for analysis on a column packed with 2%OV-17 on Chromosorb G and coupled through a Watson-Biemann He separator to the mass spectrometer.

6.6 Biological Assays

Heinert, identified hexestrol in milk using the mouse uterine weight bioassay method (39). Rennet

coagulation of the milk permitted detection of 0.001 ppm of hexestrol which could also be detected in cheese after coagulation of milk. Liem etal (40) described a biological assay of estrogenic substances in cosmetic including hexestrol, based on application of the cosmetic products to the shaven skin of castrated female mice; vaginal smears were taken subsequently for analysis.

Acknowledgements

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MESTRANOL

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1. Description

1.1 Nomenclature

1.11 Chemical Names

Ethinylestradiol-3-methyl ether.
 17 α -Ethinylestradiol-3-methyl ether
 3-Methoxy-19-nor-17 α -pregna-1,3,5(10)-trien-20-yn-17-ol.
 19-Norpregna-1,3,5(10)-trien-20-yn-17-ol, 3-methoxy-(17 α)-
 17 α -Ethinyl-3-methoxy-1,3,5(10)-estratrien-17 β -ol.
 17 α -Ethinyl-3-methoxyoestra-1,3,5(10)-trien-17-ol.
 17 α -Ethinyl-1,3,5(10)-estratriene-3, 17 β -diol-3-methyl ether.

1.12 Generic Name

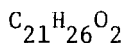
Mestranol.

1.13 Trade Names

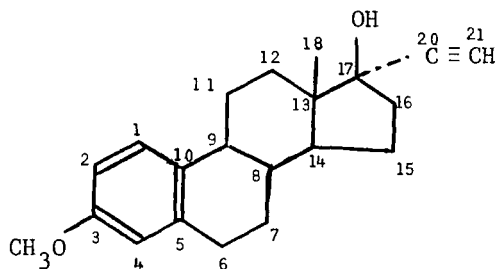
Mestranol is an ingredient of the following proprietary oral contraceptive preparations:
 Conovid, C-Quens, Enavid, Enovid, Metrulen, Norinyl, Previson, Ortho-Novum, Ovanon, Ovulen, Sequens, Syntex Menophase.

1.2 Formulae

1.21 Empirical



1.22 Structural



1.23 CAS No.

72-33-3

1.3 Molecular Weight

310.42

1.4 Elemental Composition

C 81.25%, H 8.44%, O 10.31%.

1.5 Appearance

White crystalline powder.

2. Physical Properties2.1 Crystal Data

Crystal data were reported by Ohrt et al (1) for some esterone-related compounds. The data given for mestranol was : a 6.998, b 39.737, c 6.871A., β 117.58°, P_{21} , Z = 4.

2.2 Melting Point

Melts between 146° and 154° with a range of 4°(2,3).

2.3 Solubility

Almost insoluble in water, soluble 1 in 44 of ethanol, 1 in 23 of ether, and 1 in 4.5 of chloroform, 1 in 12 of dioxane, 1 in 23 of acetone, slightly soluble in methanol (2,4,5).

2.4 Identification2.41 Infrared Spectroscopic Test

B.P. (4) and U.S.P. XIX (2) make use of the infrared absorption spectrum of mestranol as a mean of identification of the drug. The infrared absorption spectrum of the sample exhibits maxima which are only at the same wavelengths as, and have similar relative intensities to, those in the spectrum of a standard mestranol. The infrared spectrum of mestranol will be discussed later

in the spectral properties of the drug.

2.42 Ultraviolet Spectroscopic Test

U.S.P. XIX requires that for the identification of the drug, the ultraviolet absorption spectrum of methanolic solution exhibits maxima and minima at the same wavelengths as that of a similar solution of a standard mestranol, concomitantly measured. The ultraviolet spectrum of mestranol will be discussed later in the spectral properties of the drug.

2.43 Thin Layer Chromatographic Test

B.P. and U.S.P. XIX describe a thin layer chromatographic method for the identification of mestranol in which the principal spot in the chromatogram of the substance being examined is compared with that of mestranol obtained under identical conditions.

2.44 Color Test

According to B.P. a solution of mestranol in sulfuric acid appears orange-red by transmitted light, shows a yellowish-green fluorescence by reflected light and produces a reddish-brown flocculent precipitate after addition of ferric ammonium sulfate solution and a rose-red flocculent precipitate when water is added.

2.45 Solubility Test

B.P. uses a solubility test to distinguish mestranol from ethinylestradiol. The former is insoluble in a 5% w/v solution of potassium hydroxide.

2.5 Spectral Properties

2.51 Ultraviolet Spectrum

The ultraviolet absorption spectrum of mestranol obtained from a solution in neutral methanol in the region of 200 to 350 nm using a Varian Cary 219 spectrophotometer is shown in Figure I. Two absorption maxima at about 278 and 286 nm and

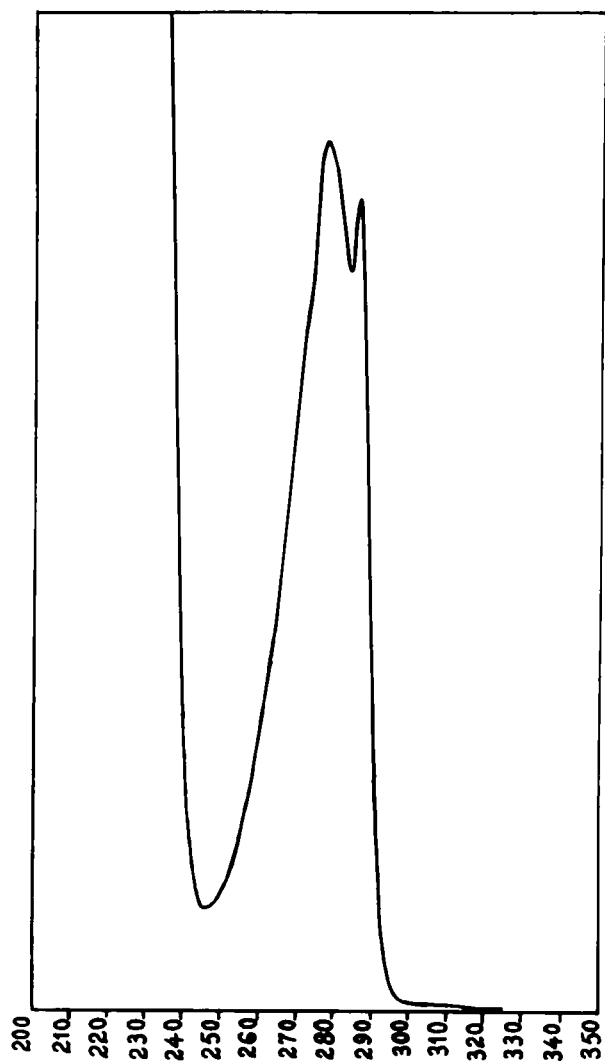


Fig. 1. Ultraviolet spectrum of mestranol in neutral methanol.

two minima at about 246 and 284 nm were observed. Reported (5) ultraviolet absorption spectrum of mestranol in methanol exhibited two maxima at 279 nm (E 1%, 1 cm 82) and 287.5 nm (E 1%, 1 cm 14.4).

2.52 Infrared Spectrum

The infrared spectrum of mestranol is presented in Figure 2. The spectrum was obtained from nujol mull using a Unicam SP 3-300 infrared spectrophotometer. The spectral assignments are presented in Table 1.

Table 1. Infrared spectral assignments for mestranol.

<u>Band Frequency₁</u> <u>Wavenumber cm</u>	<u>Structural assignment</u>
3500	O-H stretching.
3300	\equiv CH stretch.
1510, 1620	-C=C-stretch of the aromatic ring.
1460	C-H Deformation of $-\text{CH}_2-$
1380	-C-H Deformation of $-\text{C}-\text{CH}_3$
1300	C-O-C stretch.
653	\equiv C-H Deformation.

2.53 Proton Magnetic Resonance (PMR) Spectrum.

The PMR spectrum of mestranol is presented in Figure 3. The sample was dissolved in CDCl_3 and the spectrum obtained on a Varian-T60A NMR spectrometer with tetramethylsilane as the internal standard. The spectral assignments shown in Table 2, are in agreement with reported studies (6).

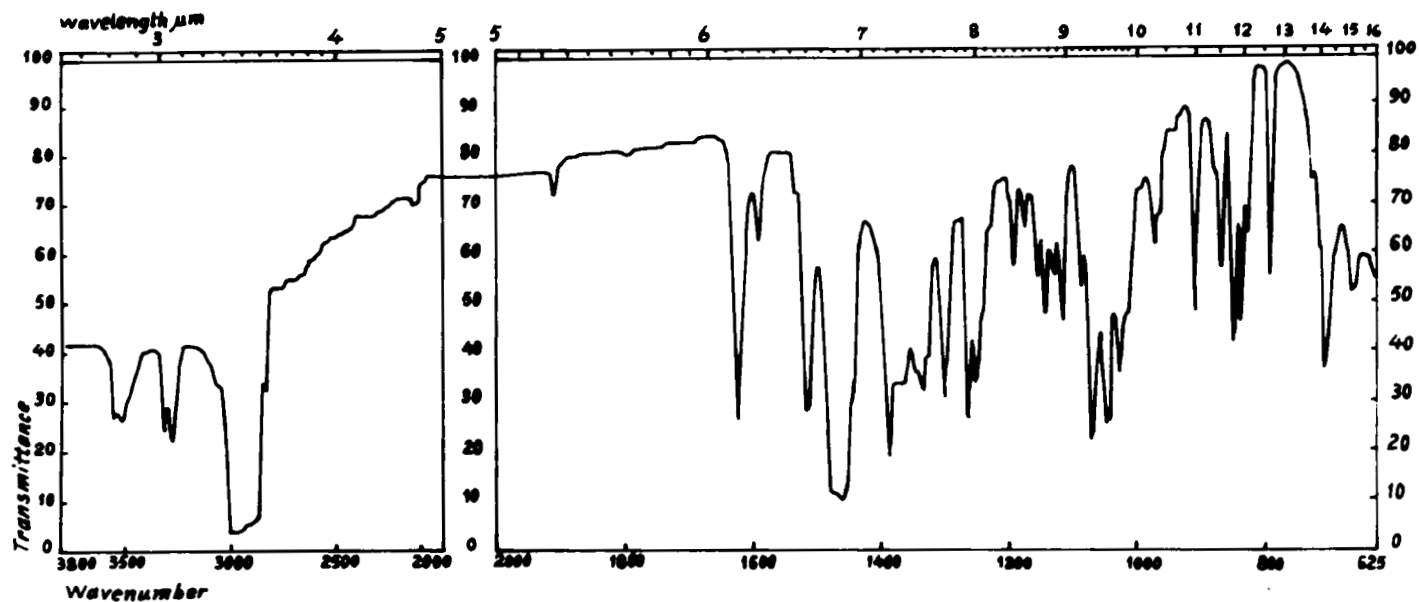


Fig. 2. Infrared spectrum of mestranol; Nujol Mull.

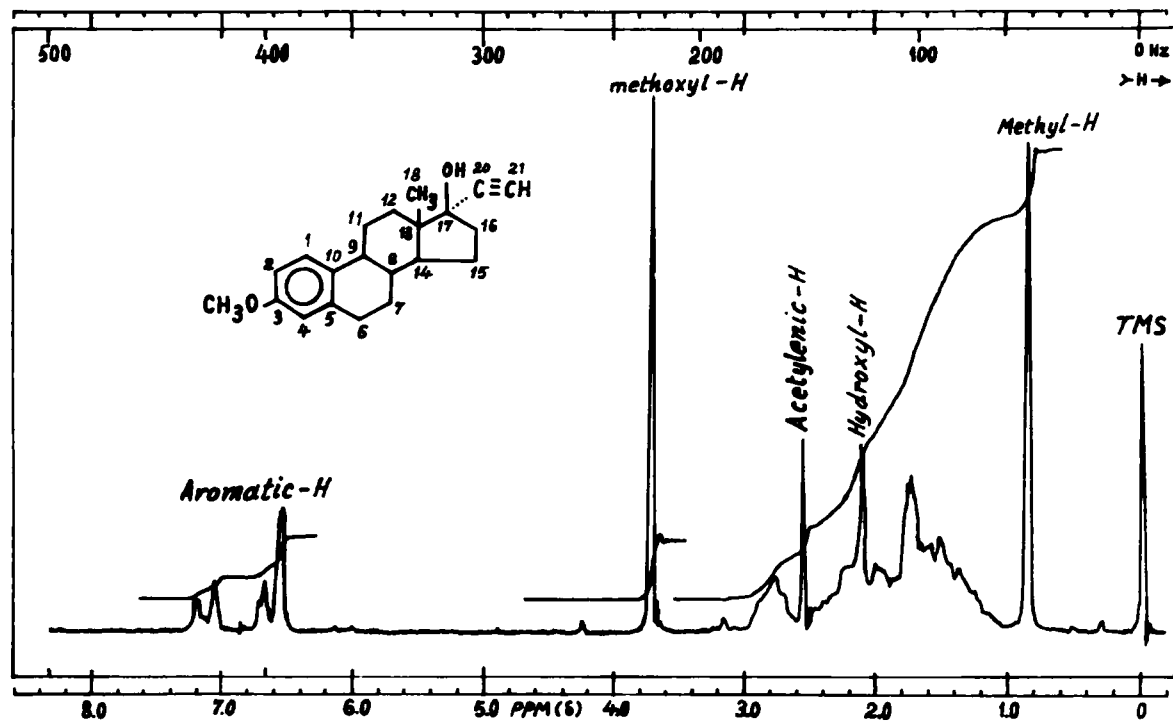


Fig. 3. PMR spectrum of mestranol in CDCl_3 with TMS internal standard

Table 2. PMR spectral assignments for mestranol.

Chemical Shift. ppm(δ)	Multiplicity	Number of Protons.	Species
0.92	singlet	3	-CH ₃
2.12	singlet	1	-OH
2.55	singlet	1	acetylenic proton.
3.72	singlet	3	methoxy protons.
6.87	multiplet.	3	Aromatic protons.

2.54 ¹³C - Nuclear Magnetic Resonance (¹³C NMR) Spectrum

The ¹³C NMR spectrum of mestranol in CDCl₃ using tetramethylsilane as an internal standard reference was obtained on a Jeol FX 100, 100 MHz instrument at an ambient temperature using a 10mm. sample tube. The spectrum is presented in Figure 4 and the chemical shift values, derived from the off-resonance spectrum, is shown in Table 3.

Table 3. ¹³C NMR spectral assignments for mestranol.

Carbon No.	Chemical shift. (ppm)	Carbon No.	Chemical shift. (ppm)
1	126.2590	13	47.2199
2	113.9788	17	79.9181
3	157.5908	18	12.7185
4	111.5932	20	87.7616
5	137.9037	21	73.9221
10	132.6407	CH ₃ -O-	55.2115

2.55 Mass spectrum and Fragmentometry

The mass spectrum of mestranol, obtained by electron impact ionization, using Nermag GC-Mass spectrometer model R 1010, is presented in Figure 5. The spectrum shows a molecular ion M⁺ at m/e 310 (relative intensity 24.4%) and a base peak

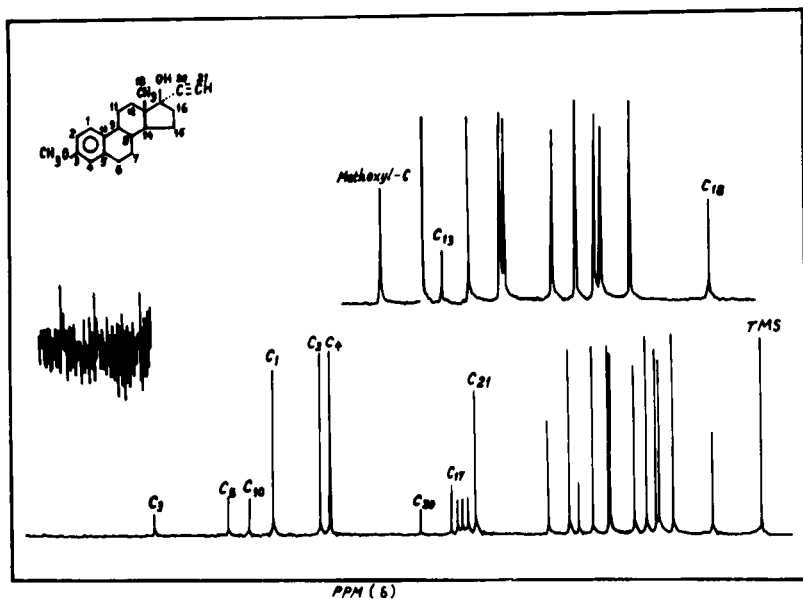


Fig. 4. ^{13}C NMR spectrum of mestranol in CDCl_3 with TMS internal reference.

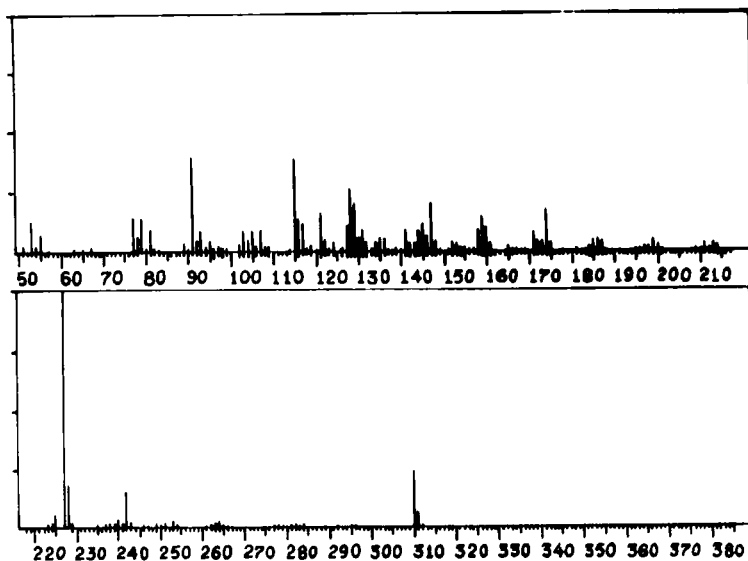


Fig. 5. Mass spectrum of mestranol (EI).

at m/e 227. Based on the data interpretation and summary (INTSUM) program presented by Smith *et al.*, (7) for the possible fragmentation of the basic skeleton of estrogenic steroids, a proposed fragmentation pattern of mestranol is shown in Table 4.

Table 4. Proposed mass fragmentation pattern of mestranol.

<u>m/e</u>	<u>Relative intensity %</u>	<u>ion</u>
311	6.3	M ⁺¹
310	24.4	M ⁺
242	15.7	C ₁₆ H ₁₈ O ₂ ⁺
228	19	C ₁₆ H ₂₀ O ⁺
227	100	C ₁₅ H ₁₅ O ₂ ⁺
160	10	C ₁₁ H ₁₂ O ⁺
159	14.6	C ₁₁ H ₁₁ O ⁺
147	20.5	C ₁₀ H ₁₁ O ⁺
145	11.9	C ₁₀ H ₉ O ⁺
129	20.7	C ₁₀ H ₉ ⁺
128	26.8	C ₁₀ H ₈ ⁺
116	13.9	C ₉ H ₈ ⁺

3. Synthesis

Mestranol was prepared by Colton *et al.* (8) as follows: Estrone {I} is converted to its 3-methoxy analog {II} by reaction with methyl sulfate. The ethynyl group may then be introduced at position 17 either through reaction with sodium acetylide in liquid ammonia followed by hydrolysis of the sodoxy compound, or through Grignardization with ethynyl magnesium bromide. Almost the sole product of the ethynylation reaction is that which results from attack of reagent from the least hindered α -side of the steroid, Fig.6.

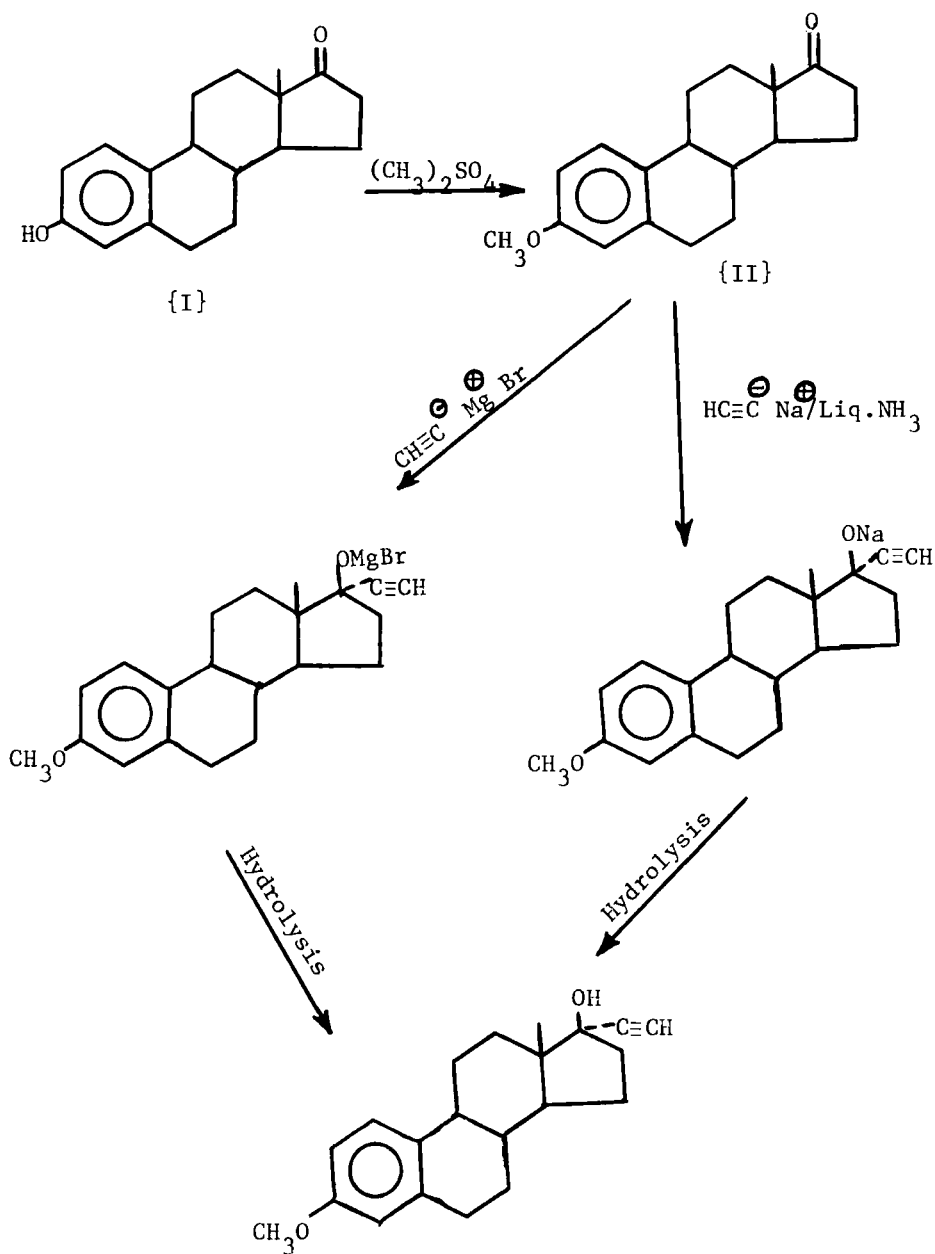


Figure 6. Synthesis of mestranol.

4. Absorption, Metabolism and Excretion

Mestranol is a synthetic estrogen which is more potent than estradiol. It is readily absorbed from the gastrointestinal tract and is slowly metabolized and excreted in urine and feces. The absorption, metabolism and excretion of the drug has been extensively studied in animals and humans, Fig.7.

Wijmenga and Van der Molen (9) reported a biological half-life of mestranol of 50 hours and that a small proportion of the drug was excreted in milk of nursing mothers.

Mills et al, (10) showed that after the injection of tritium-labelled mestranol to women, the radioactivity representing the metabolites of the drug in the blood disappeared with an average half-life of 45 hours (range 37-65 hours). Mahesh et al, (11) studied the metabolic clearance rate and blood half-life of mestranol. The metabolic clearance rate of the drug after a single i.v. injection to women was 1265L/day. The radioactivity half-life after injection was 45.1 hours. No change in the metabolic clearance rate of the drug was noticed for upto 7 months of oral contraceptive medication. Using the constant infusion technique, Bird & Clark (12) measured the metabolic clearance rate of mestranol in normal young women and found it to be 1741L/24 hours. The mean conversion ratio for mestranol to ethynylestradiol was 0.236 and to ethynylestradiol sulfate 3.369; that of mestranol to the sulfate was 6.476. The mean transfer constant for mestranol to ethynylestradiol was 0.182. The principal circulating form of the drug was ethynylestradiol sulfate.

Mills et al (13) administered mestranol orally to normal women in order to measure the metabolic clearance rate and to study the urinary excretion of the drug. Mestranol was rapidly cleared from the plasma with an average metabolic clearance rate of 1247L/day. About 30% of mestranol administered was excreted in urine in 5 days, less than 4% in an unconjugated form, 10% as sulfate conjugates and about 5% as glucuronide conjugates. Comparison of the metabolic clearance and urinary excretion rates made before and again after six cycles of treatment with Ortho-Novum SQ containing mestranol showed that prolonged administration had no effect on the metabolic clearance rate, rate of urinary excretion or mode of conjugation of the drug or its metabolites.

In a study by Bolt and Remmer (14) following the i.v. administration of ^{14}C -labelled mestranol to female rats; 45 and 3% of the radioactivity were found to be excreted in the feces and urine, respectively, in 3 days. Only 2.5% of the radioactivity was expired as $^{14}\text{CO}_2$ within 4 days indicating no significant degradation of the steroid nucleus. Some metabolites separated from fecal extracts had an unaltered 3-methoxy group. Others, however, were demethylated to derivatives of ethynylestradiol.

The chronic i.v. injection of radioactive mestranol into mice resulted, as reported by Bolt and Remmer (15), in an accumulation of radioactivity in organs to a greater extent than activity accumulated after radioactive estradiol administration. The metabolites of mestranol were tightly bound to the liver tissue and were not removed by solvent extraction or by acid hydrolysis. The high demethylation rate of mestranol in mice as compared to that in rats may be due to a high activity of the microsomal oxidase in the mouse liver. Following a single i.v. injection of the steroid into female rats, 1.5% of the radioactivity was recovered from urine collected in the first 3 days, and 55% of the activity was excreted in the feces within 3 weeks.

Hanasono and Fischer (16) studied the excretion of tritium-labelled mestranol and other contraceptive steroids and the enterohepatic circulation (EHC) of their metabolites in female rats. Mestranol was rapidly and extensively eliminated as metabolites in the bile after a single i.v. dose. The cumulative percentage of administered radioactivity appearing in the bile at the end of 8 hours was 69%. Mestranol appeared in the bile primarily as a glucuronide conjugate and other polar materials which were not sulfate conjugates. Intact female rats given single i.p. doses of the labelled steroid eliminated radioactive metabolites in the urine and feces at a much slower rate than that seen in the bile of animals with biliary fistulas. Fecal excretion was the major route of elimination in intact animals for the steroid and accounted for 80% or more of the radioactive dose by the end of 7 days. Experiments were also conducted to assess the enterohepatic circulation of metabolites of the steroid. 59% of the intraduodenally infused radioactivity associated with the biliary metabolites of mestranol, underwent enterohepatic circulation and appeared in the bile during a 24 hour period. The glucuronide conjugate fraction of biliary metabolites was the most important fraction undergoing enterohepatic circulation. The

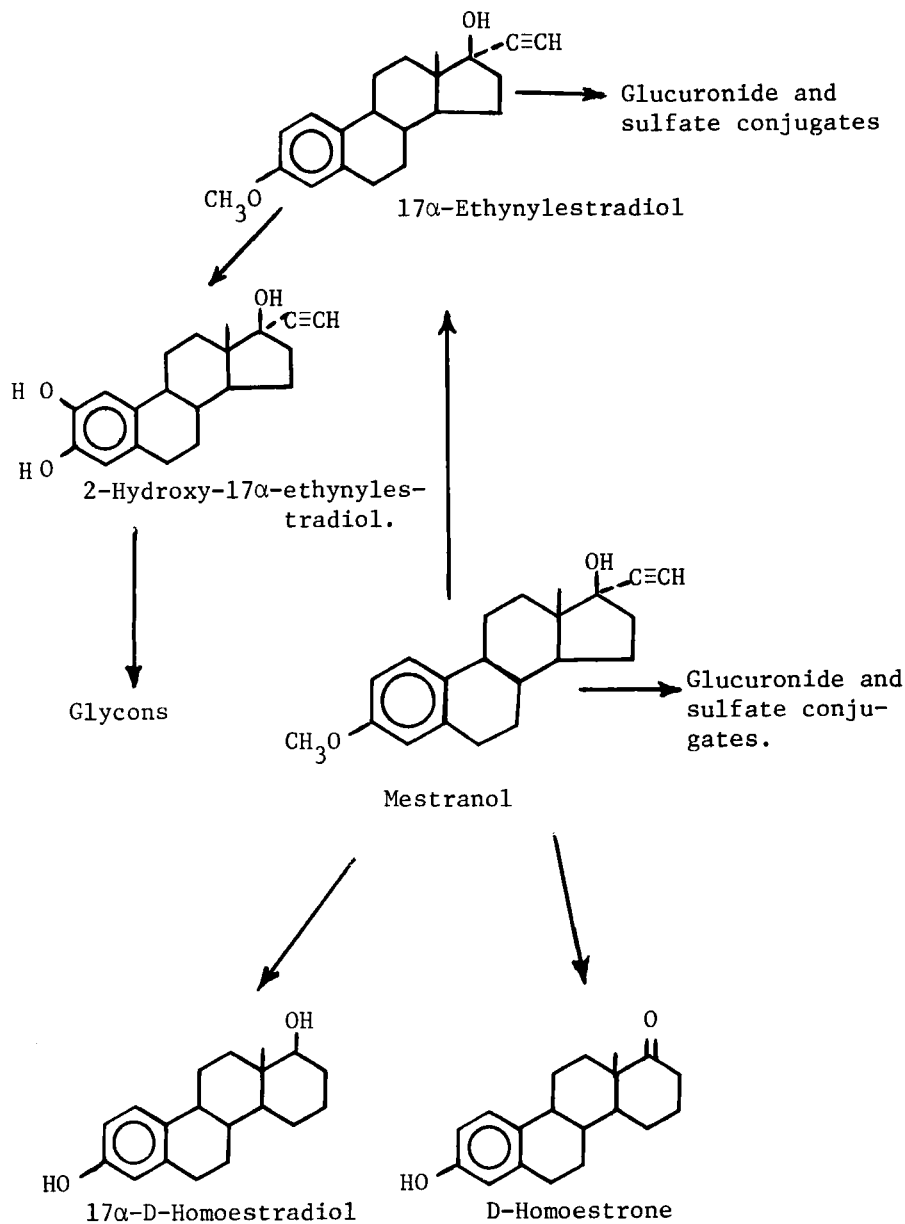


Figure 7. Metabolism of mestranol.

polar metabolites, which could not be hydrolysed by β -glucuronidase, were not absorbed from the intestine and re-excreted in the bile. The authors conclude that the enterohepatic circulation is an important feature of the disposition of the contraceptive steroid in rats and is dependent on the excretion of glucuronide conjugates of the steroid metabolites in the bile.

In a study using radioactive mestranol, Williams (17) identified 17 α -ethynylestradiol as a urinary metabolite using reverse isotope dilution technique.

Abdel-Aziz and Williams (18), studying the urinary metabolites of mestranol in guinea pigs, identified 17 α -ethynylestradiol conjugates, D-homoestradiol-17 β and D-homoestrone, as metabolites.

In their study of the metabolism of 4-³H and 4-¹⁴C-mestranol by women, Williams et al (19), reported that reactions involving position 4 were no greater than 1.7 to 3% of the dose as measured by the liberation of ³H into body water. The extent of deethynylation in vivo was no greater than 1 to 2% of the dose as measured by urinary estrone metabolites. Mestranol (0.7 and 0.32% of the dose), 17 α -ethynylestradiol (6.6 and 11.3%) and 2-hydroxy-17 α -ethynylestradiol (0.64 and 0.7%) were identified as metabolite glycons by reverse isotope dilution after ketodase hydrolysis of the urine from two of the women.

5. Methods of Analysis

5.1 Titrimetric Methods

- (a) The B.P. (4) recommended a titrimetric method in which the drug in tetrahydrofuran is treated with silver nitrate and then titrated with standard sodium hydroxide solution. The end point determined potentiometrically.
- (b) An argentimetric determination of some acetylene steroidal drugs, including mestranol, had been developed by Roushdi et al (20). The assay method depends on the precipitation of the steroid silver salt from alcoholic ammoniacal silver nitrate solution followed by extraction with a suitable organic solvent, evaporation of the solvent and then applying Volhard's method.

- (c) Roushdi et al (21) used a nonaqueous titrimetric method for determination of acetylenic steroids. The method involves the precipitation of the steroid silver salt using ammoniacal silver nitrate solution. The salts extracted will chloroform or ether and titrated with perchloric acid in acetic acid using Gentian Violet as indicator.

5.2 Spectrophotometric Methods

5.21 Ultraviolet Spectrometric Methods

- a) Bastow (22) determined mestranol from its absorption at 280 nm after elimination of the interfering ketonic absorption by reduction with borohydride. Residual interference was allowed for by a three point correction.
- b) Gorog and Csizer (23) described methods for the determination of (i) mestranol as an impurity in norethynodrel and norethisterone and (ii) mestranol in contraceptive tablets. For (i) the sample is dissolved in methanol and treated with sodium borohydride. A solution of mestranol is treated similarly. The extinctions of the two solutions are measured at 287, 290.5 and 294 nm, and the mestranol content is calculated from a given equation. For (ii) the powdered tablets are heated under reflux with dichloromethane. After filtration and evaporation of the solvent, mestranol is determined as in (i). The mean recovery for the latter method was $98.3 \pm 2.3\%$.
- c) A specific spectrophotometric method for the determination of 17-ethynyl steroids was published by Szepesi and Gorog (24). 17 α -Ethynyl steroids were determined by quantitative conversion to 17-oxo steroids by sodium t-butoxide at 81 $^{\circ}$, followed by spectrometric determination of the 16-glyoxalyl derivatives, λ_{max} . 294 nm, ϵ 10,700, standard deviation 1.1%. By the same principle, at 0 $^{\circ}$, the 17-oxo impurity content in 17-ethynyl steroids was determined. The method was not applicable to 3-oxo steroids.

- d) Shroff and Grodsky (25) described an ultraviolet spectrophotometric method for the estimation of mestranol in fresh tablets. In this method the sample is shaken with water and methylcyclohexane, centrifuged and the extinction of the upper phase measured at 287.7 nm. Base-line corrections applied are calculated from (i) the extinction at 302 nm and (ii) the extinctions at 278 nm and 302 nm and the extinction ratio E_{278} to $E_{287.7}$ for pure mestranol; the two corrected values of $E_{287.7}$ for the sample should agree within $\pm 0.006\%$. The contents of mestranol is calculated from the mean corrected value. The coefficient of variation for ten samples is 1.14%. The method is not preferred for aged tablets because on storage an interfering complex is formed between poly (vinyl pyrrolidone) and magnesium stearate excipients.

5.22 Colorimetric Methods

- a) Chlorine o-tolidine reagent was used by Huettenrauch (26) for the selective detection of steroids with aromatic ring A and C_{13} -OH group whether this was free, esterified or ether-linked. Mestranol gave positive reaction with sensitivity of 1-2 $\mu\text{g/cc}$.
- b) Shroff and Huettemann (27) developed a colorimetric method for assaying mestranol in tablets. The method is based on the formation of a colored complex with phenol-sulfuric acid reagent. This complex exhibits an absorption maximum of 550 nm, obeys Beer's Law and is stable for reasonable length of time. According to the method the tablets are moistened with water and shaken with methylcyclohexane. An aliquot of the organic phase is evaporated in an atmosphere of nitrogen and treated with phenol-sulfuric acid reagent and the extinction measured at 550 nm against a reagent blank. The error and precision are $\pm 1.6\%$ and 1.52% respectively. The presence of norethisterone and excipients do not interfere.

The method is claimed to be superior to the ultraviolet spectrophotometric method described by Shroff and Grodsky and summarized above.

- c) A direct colorimetric method for the assay of mestranol in one tablet is reported by Comer et al (28). In this method one tablet is dissolved in sulfuric acid/methanol (7:3) and the extinction measured at 545 nm against a reagent blank. To avoid interference by chlor-madinone, the temperature must be kept below 5° during the color reaction. The coefficient of variation was 1.05%. Very similar methods have also been described by Templeton et al (29) for one tablet assay and by Wu (30) for mestranol in combination with ethynodiol diacetate. The latter procedure require prior chromatographic separation of mestranol.
- d) Beyer (31) developed an automated colorimetric procedure for the quantitation of mestranol in tablets according to which the tablets are suspended in water and extracted with chloroform-ethanol. In an automatic system, re-extraction is effected with 10% ethanol solution in 90% sulfuric acid. The extinction is measured at 538 nm. The coefficient of variation is $\pm 1\%$.
- e) Rizk et al (32) determined mestranol colorimetrically by means of silver ions. An ethanolic solution is treated with aqueous ammonia and an excess of aqueous silver nitrate and the precipitated silver compound is filtered off. Excess silver ions in the filtrate is determined by a dithizone spectrophotometric procedure and the amount of steroid is calculated from the silver ions consumed.

5.23 Infrared Spectroscopic Methods

- a) Mestranol was determined in chloroform solution by infrared spectrophotometry (33) at 3308 cm^{-1} (Ethynyl vibrations) or 1502 cm^{-1} (aromatic system vibrations).

- b) Chatten et al (34) reported a quantitative assay of antifertility agents by infrared spectroscopy. Two pure estrogenic substances and eight pure progestogens were analysed in the selected wavelength range and shown to obey Beer-Lambert Law. Although progestogen analysis in compound tablets was accomplished in several cases using the technique, the estrogenic content in all compound tablets was too low to permit analysis.
- c) Beyermann and Roder (35) analysed mestranol in oral contraceptives by infrared spectrophotometry after separation of the components by thin-layer chromatography. The separation was performed on silica gel with ethyl acetate as a solvent. The appropriate spot extracted with chloroform and the infrared spectrum in liquid paraffin mull was recorded and mestranol determined from its extinction at 7.95 μm .

5.24 Spectrofluorometric Methods

- a) Cullen et al (36) developed a sensitive procedure for the analysis of norgestrel and structurally related steroids based on sulfuric acid induced fluorescence. The selectivity of the reaction and mechanism of fluorescence formation were studied. The reaction is specific for Δ^4 -3-ketosteroids which have both a 17 β -hydroxy and a 17 α -alkyl or alkyne substitution and $\Delta^{1,3,5(10)}$ -triene-3-ol steroids. A two-step mechanism was tentatively explained on the basis of the effects of temperature, time, initial acid concentration and subsequent dilution with water on fluorogen development. The procedure has been automated to permit unit dose analysis.
- b) A spectrofluorometric procedure for the determination of mestranol in some oral contraceptive tablets had been developed (37) which utilizes the native fluorescence of mestranol. The method is claimed to be rapid, reliable and sensitive. No separations are required and the method is applicable in the presence of norethynodrel, ethynodiol diacetate and norethisterone,

but chlormadinone acetate interferes. The method is applicable to single-tablet analysis. According to the procedure one powdered tablet is shaken with anhydrous ethanol and centrifuged. An aliquot of the supernatant is diluted with ethanol and the fluorescence at 327 nm (excitation at 284 nm) is compared with that of a standard solution. Anhydrous ethanol is used as a reference solution and a correction applied for its fluorescence. The accuracy under the conditions studied was $\pm 1.29\%$ and the precision ranged from ± 0.896 to ± 1.73 .

- c) Mariani and Mariani-Vicari (38) also described a method for the assay of mestranol spectrofluorometrically. A sulfuric acid-methanol (1:1) reagent is used and a previous separation of the two components (ethynylestradiol and mestranol) in the compound preparation is not required. For tablets the hydrolysis of the starch by diastase is followed by extraction into chloroform. The wavelengths of emission and excitation spectra are 515 nm and 475 nm respectively.
- d) An automated fluorimetric method for one-tablet assay of mestranol was carried out by Comer et al (28). One tablet is dissolved in sulfuric acid-methanol (7:3) and the fluorescence measured with Kodak Wratten No.58 ~~and~~ Corning 1-60 primary filters and a Kodak Wratten 22 secondary filter. A scheme for automation of the fluorimetric method is illustrated. In twelve replicate experiments, the coefficient of variation was 1.30%. The fluorescence reaction is retarded by NO_3 , NO_2 and H_2O_2 . Related steroids, such as estradiol methyl ether, esterone methyl ether and esterone, cause little interference.
- e) Templeton et al (29) described a fluorimetric assay method of mestranol in which one tablet is allowed to disintegrate in 20% sodium hydroxide solution and water, then extracted with chloroform and an aliquot of the extract evaporated to dryness. The residue dissolved in 50% methanol solution in concentrated sulfuric acid and the fluorescence measured at 498 nm, with excitation at 468 nm.

- f) A fluorimetric assay method for the determination of mixture of mestranol and norethynodrel was reported by Pastor *et al* (39). For mestranol alone extracts (in 1:5 chloroform-methanol) were evaporated to dryness, dissolved in ethanol cooled, and allowed to react with a mixture of 1:3 acetic acid-sulfuric acid at room temperature before measuring the fluorescence emission at 560 nm after excitation at 545 nm. When norethynodrel was present with mestranol, the extracts were treated with borohydride in methanol at room temperature before addition of concentrated hydrochloric acid and processing as above to estimate mestranol. Norethynodrel was estimated in the mixture, from borohydride and acetic acid-sulfuric acid treatment, by excitation at 485 nm and emission at 520 nm. Variation in measurements were 3% for mestranol and 2% for norethynodrel.
- g) Mestranol in oral contraceptive tablets was also determined fluorimetrically (40) by disintegrating a tablet in dilute hydrochloric acid, extracting with methylene chloride, treating with 0.2% hydroquinone (in 70:30 sulfuric acid-ethanol) and measuring the fluorescence.
- h) Dusinsky and Radejova (41) reported fluorimetric methods for the determination of mestranol and some other estrogenic hormones. Methods, based on the determination of 'native' fluorescence and that 'induced' by sulfuric acid, for determining mestranol, ethinylestradiol, estradiol benzoate and valerate and the total estrogenic hormone content in mixtures of natural conjugated estrogens are described. The methods are claimed to be simple, highly sensitive and rapid. There is no need for preliminary clean-up or hydrolysis.

5.25 Nuclear Magnetic Resonance (NMR) Spectrometric Methods

Avdvich *et al* (6) used an NMR technique to quantitate mestranol bulk drug. Diphenylacetic acid was used as the internal standard and pyridine as the solvent. The amount of mestranol was calculated from the integrals of the peaks at 6.33 ppm

(methoxyl protons) and at 6.80 ppm (ethynyl proton de-shielded by pyridine). The average deviation was $\pm 0.6\%$ and the results were in good agreement with those obtained by an official method.

5.3 Chromatographic Methods

5.31 Thin-Layer Chromatography

The literature describes several thin-layer chromatographic methods for the separation and analysis of mestranol in mixtures and contraceptive tablets. For quantitation, the plate is first scraped and mestranol eluted before treatment with a suitable reagent. Table 5 summarizes thin-layer chromatographic methods for mestranol.

5.32 Column Chromatography

- a) Quantitative separation of progestins and estrogens from anovulatory formulations had been performed (57) using gel filtration on a synthetic polysaccharide (Sephade LH-20) and the compounds were determined directly by U.V. spectrophotometry.
- b) Brunner and Kunze (58) published an analytical method for mestranol in combination with norethisterone (norethinadrone) or norethynodrel by partition chromatography and ultra-violet measurement. According to this procedure oral contraceptive tablets were extracted with dimethylformamide-formamide (1:1) and the steroids separated by partition chromatography with use of this solvent as stationary phase on Celite with heptane as mobile phase. Mestranol, in the relevant fraction, is determined by measuring the extinction at 287 nm with base-line correction obtained from the extinction at 302 and 315 nm. Interference was noticed with ethynodiols diacetate and chlormadinone acetate. The method had been adopted as official first action after a collaborative study was conducted (59).
- c) A method was described (60) for the separation of steroids in mixtures of pharmaceutical interest by means of silicic acid column chro-

Table 5: Thin-Layer Chromatographic systems for mestranol analysis.

<u>Stationary Phase</u>	<u>Developing solvent</u>	<u>Visualization</u>	<u>Quantitative</u>	<u>Reference</u>
Silica Gel or Neutral Allumina		0.5% Vanillin H_2SO_4 -EtOH	-	42
Kieselgel G	Cyclohexane/EtoAc (7:3)	-	Treat with 40% SbCl_3 in HoAc measure extinction at 570 nm or fluorescence.	43
Silica Gel G/H	Ether/Cyclohexane (8:2)	Iodine vabors	Treat with 15% TiCl_3 in HoAc- H_2SO_4 , measure extinction at 530 nm.	44
Propylene glycol- impregnated Kaie- selguhr G.	Toluene, Cyclohexane- toluene (8:2) or pet- roleum ether.	20% p- $\text{MeC}_6\text{H}_4\text{SO}_3\text{H}$ in 94% EtOH, heat at 120 for 10 min.	-	45
Paraffin oil-imp- regnated Kieselguhr G.	30,50 or 70% HoAC solutions.	Same as above.	-	45
Silica Gel	Benzene/ethyl methyl ketone (9:1).	-	Treat with H_2SO_3 -MeOH(2:1) measure extinction at 540 nm.	46

contd.....

<u>Stationary Phase</u>	<u>Developing solvent</u>	<u>Visualization</u>	<u>Quantitative</u>	<u>Reference</u>
Kieselgel G/HR				
a) 1-Dimensional	EtoAc/cyclohexane/Me ₂ CO (5:15:2)	85% Phosphoric- MeOH(1:1) or SbCl ₃ -HoAc (1:1 w/v), heat at 120°-130°.	Use Kieselgel HR Treat with 15% TiCl ₃ - conc. HCl. Measure extinction.	47,48
b) 2-Dimensional	Above solvent followed by Cyclohexane/EtoAc (23:27).	or Iodine vabors.		47,48
Silica Gel GF	2-Dimensional: Chloroform/methanol (9:1) or Benzene/acetone(95:1) followed by benzene/ methanol (95:5) or methylene chloride/methanol /water (150:9:0.5)	H ₂ SO ₄ and heat for 30 minutes at 100°. UV irradiation.	Visual comparison of 49 of the spots sizes and color intensi- ties with standard spots.	
Kiesel G	Benzene	Iodine vabors	-	50
Silica gel G	Heptane/acetone(4:1)	EtOH-conc. HCl(49:1)	Extract in propanol 51 plus H ₂ SO ₄ -H ₂ O(7:3) measure extinction at 545 nm.	
Silica gel	-	HCl gas and UV irradiation.	-	52

contd.....

<u>Stationary phase</u>	<u>Developing solvent</u>	<u>Visualization</u>	<u>Quantitative</u>	<u>Reference</u>
Silica gel G, Al ₂ O ₃ GF ₂₅₄ and Silica gel GF ₂₅₄	Petroleum ether/benzene/ He ₂ CO (5:4:1)	Iodine vabors, 2,4- dinitro-phenylhydra- zine or 2% H ₂ SO ₄ in EtOH.	-	53
Silica gel G-MN	Petroleum ether/Me ₂ CO	50% H ₂ SO ₄	-	54
Silica gel 60 F ₂₅₄	2-Dimensional: Toluene-95%/ethanol(9:1) followed by BuOAc/Light petroleum/AcOH(70:30:1)	H ₂ SO ₄	-	55
Silica gel GF	Benzene/EtoAc (4:1)	0.5% H ₂ SeO ₃ -conc. H ₂ SO ₄ and heat at 100° for 15 min.	By spectrodensitome- try by scanning at 360 nm by reference to an internal standard.	56

matography. Association of estrogenic with progestational steroids, in some cases extended to androgens, were considered. The elution was performed with a gradient of ether in petroleum ether, obtained by means of two metering pumps. The effluent was monitored continuously by means of a hydrogen flame ionization detector, a part of the effluent being continuously drawn off to the detector. The quantitative analysis of the steroids separated by the method was performed by a U.V. spectrophotometric or a colorimetric procedure.

5.33 Liquid Chromatography

Hara and Hayashi (61) studied the correlation of retention behaviour of steroidal pharmaceuticals in polar and bonded reverse-phase liquid column chromatography. For the systematization of correlation between the chemical structures of solutes and retention behaviour in liquid column chromatography, the retention volume of the modified steroids on silica (Corasil II) and chemically bonded reverse-phase columns (Bondapak C₁₈/Corasil) were studied using various binary solvent systems. Retention parameters for the functional groups of the steroids were calculated according to Martin's additive rule. By comparing these values obtained on normal and reverse-phase columns, characteristic features of both packings with regard to solute structures and solvent systems were elaborated.

5.34 Gas Chromatography

- a) A comparison of an UV spectrophotometric assay and a gas-liquid chromatographic assay was conducted (25) for mestranol in tablets. Both methods gave results in good agreement and both are suitable for fresh tablets. In the gas-chromatographic method the column used was stainless steel (21 in x 0.25 in) of 4% of XE-60 on Diatoport S, maintained at 195°. Nitrogen was used as a carrier gas at 70-75 ml/hour. The method is preferred for the assay of aged tablets because on storage a complex between poly (vinylpyrrolidone) and magnesium stearate excipients was produced which interfered with the UV methods and was identified by

gas-liquid chromatography.

- b) Okuno and Higgins (62) reported a method for the determination of residues of mestranol and ethynylestradiol in foliage, soil and water samples. The lower limits for the detection of mestranol and its 3-hydroxy homolog, ethynylestradiol were 0.05, 0.1 and 0.01 ppm for foliage, soil and water respectively. Samples were extracted in an acid medium to free any conjugated ethynylestradiol and then cleaned up by Florisil column chromatography. Water samples were directly analysed by gas chromatography using a flame ionization detector and a column packed with OV 17 on Gas Chrom Q. Operating temperatures were 260° for column, 275° for the inlet and 290° for the detector. Further cleaning up of the soil and vegetation samples was carried out by gel permeation and Florisil chromatography. Analysis was then carried out by gas chromatography. The lower limit of detection was 100ng for each steroid.
- c) Mestranol and norethisterone were determined in estrogen-progestin contraceptive tablets containing both components (63) by extraction with ethyl acetate and a single gas chromatography, with testosterone propionate as internal standard. The recoveries of mestranol and norethisterone were 98.54 and 99.14% respectively and the precision was 1.40 and 1.74% respectively. The procedure could be applied to single tablets containing 0.05 mg mestranol and 1 mg norethisterone.
- d) Templeton et al (29) performed a gas-liquid chromatographic assay for mestranol. A tablet is allowed to disintegrate in sodium hydroxide in water, then extracted with chloroform. A dilute cholestane solution is used as an internal standard. The column used is a glass column (2 ft. x 4 mm) containing Diatoport S (80 to 100 mesh) impregnated with 6% of silicone-gum rubber W-98 operated at 205°, with He (60 to 70 ml per min.) as carrier gas and flame ionization detector.

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NOSCAPINE

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1. Description

1.1 Nomenclature

1.1.1 Chemical Names

- a- (S)-6,7-Dimethoxy-3-(5, 6, 7, 8-tetrahydro-4-methoxy-6-methyl-1, 3-dioxolo [4,5-g]isoquinolin-5-yl)-1 (3 H)-isobenzofuranone. (1)
- b- 1- α -2-methyl-8-methoxy-6,7-methylene dioxo-1-(6, 7-dimethoxy-3-phthalidyl)-1, 2, 3, 4-tetra-hydroisoquinoline. (1).
- c- 1 (3 H) Isobenzofuranone, 6, 7-dimethoxy-3-(5, 6, 7, 8-tetrahydro-4-methoxy-6-methyl-1, 3-dioxolo-[4, 5-g] isoquinolin-5-yl)-, [S-(R* S*)]. (2)
- d- (3 S)-6, 7-dimethoxy-3-[(SR)-5, 6, 7, 8-tetra-hydro-4-methoxy-6-methyl-1, 3-dioxolo [4, 5-g] isoquinolin-5-yl] phthalide. (3).

1.1.2 Generic Names

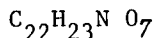
α -Gnoscapine; l- α -Narcotine; l-Narcotine; Narcotine; Noscapine.

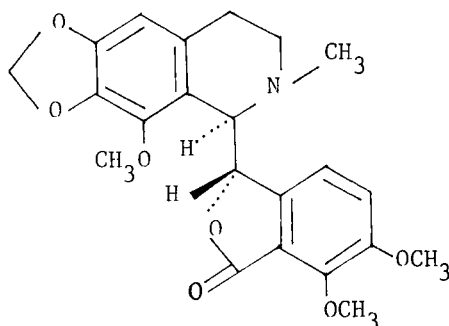
1.1.3 Trade Names

Capval; Coscopin; Coscotabs; Key-tusscapine; Longatin; Lyobex; Methoxyhydrastine; Narcompren; Narcosine; Narcotussin; Neitaclon; Nicolane; Nipaxon; Noscapal; Noscapalin; NSC 5366; Opian; Opianine; Terbenol; Tusscapine; Vadebex.

1.2 Formulae

1.2.1 Empirical



1.2.2 Structural

Noscapine

The alkaloid noscapine can be cleaved very readily into two moieties; with dilute sulfuric acid, cotarnine and opianic acid are generated. Under acidic reducing conditions, e.g., zinc in hydrochloric acid or sulfuric acid, hydrocotarnine and meconine are formed (Scheme 1). With the structural elucidation of cotarnine, opianic acid, hydrocotarnine and meconine, and given the presence of a lactone ring in noscapine, the structure of this alkaloid was essentially established (4).

1.2.3 CAS No

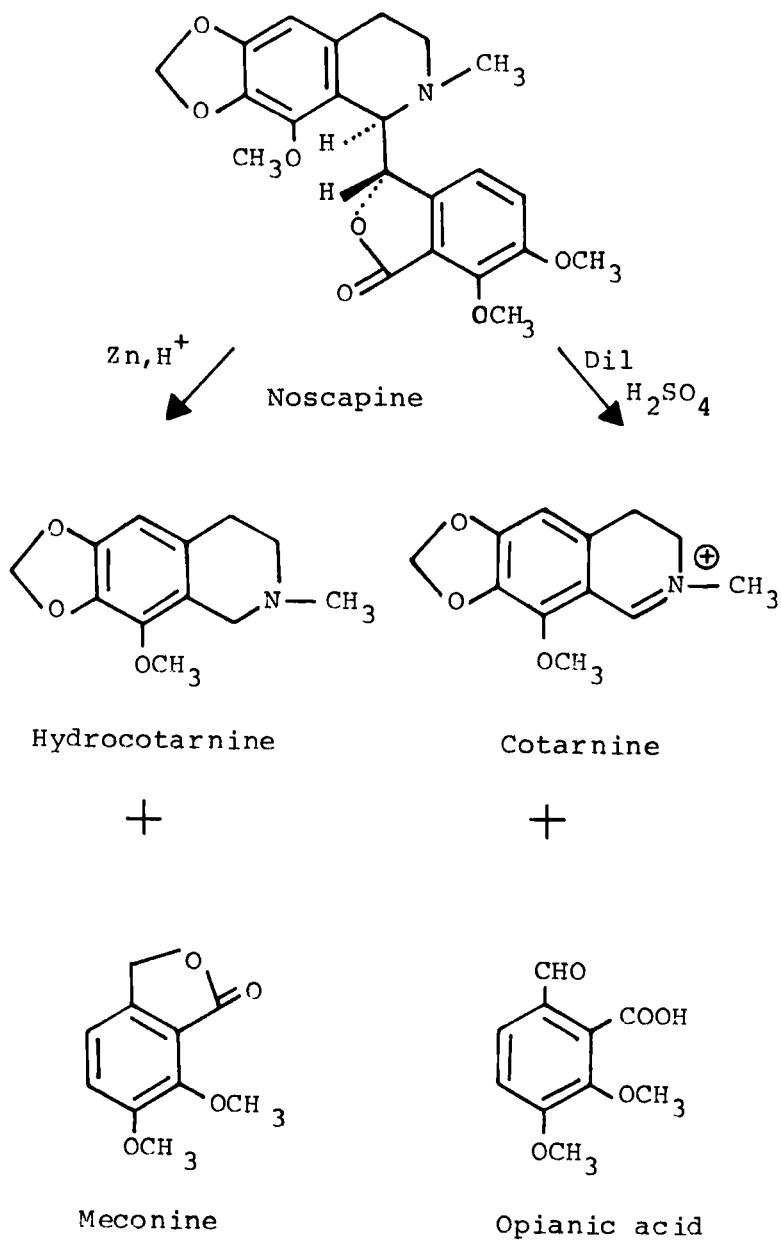
(128-62-1)

1.2.4 Wiswesser Line Notation

T C566 DO FO KN EH && TJ H
01 K J T56 B VO DHJ HOL l
01 *ALPHA* LV.

1.2.5 Stereochemistry and Absolute Configuration

The stereochemistry of noscapine has been studied by many workers (5-9). The prolonged action of hot methanolic potassium hydroxide on natural (-)- α -narcotine results in the formation of an equilibrium mixture of the original base and a new optically



Scheme 1

active diastereoisomer, (-)- β -narcotine, which can be written as shown in Scheme 2. Lithium aluminum hydride reduction of the α - and β -noscapines readily affords α -narcotinediol 1 and β -narcotinediol 2 respectively.

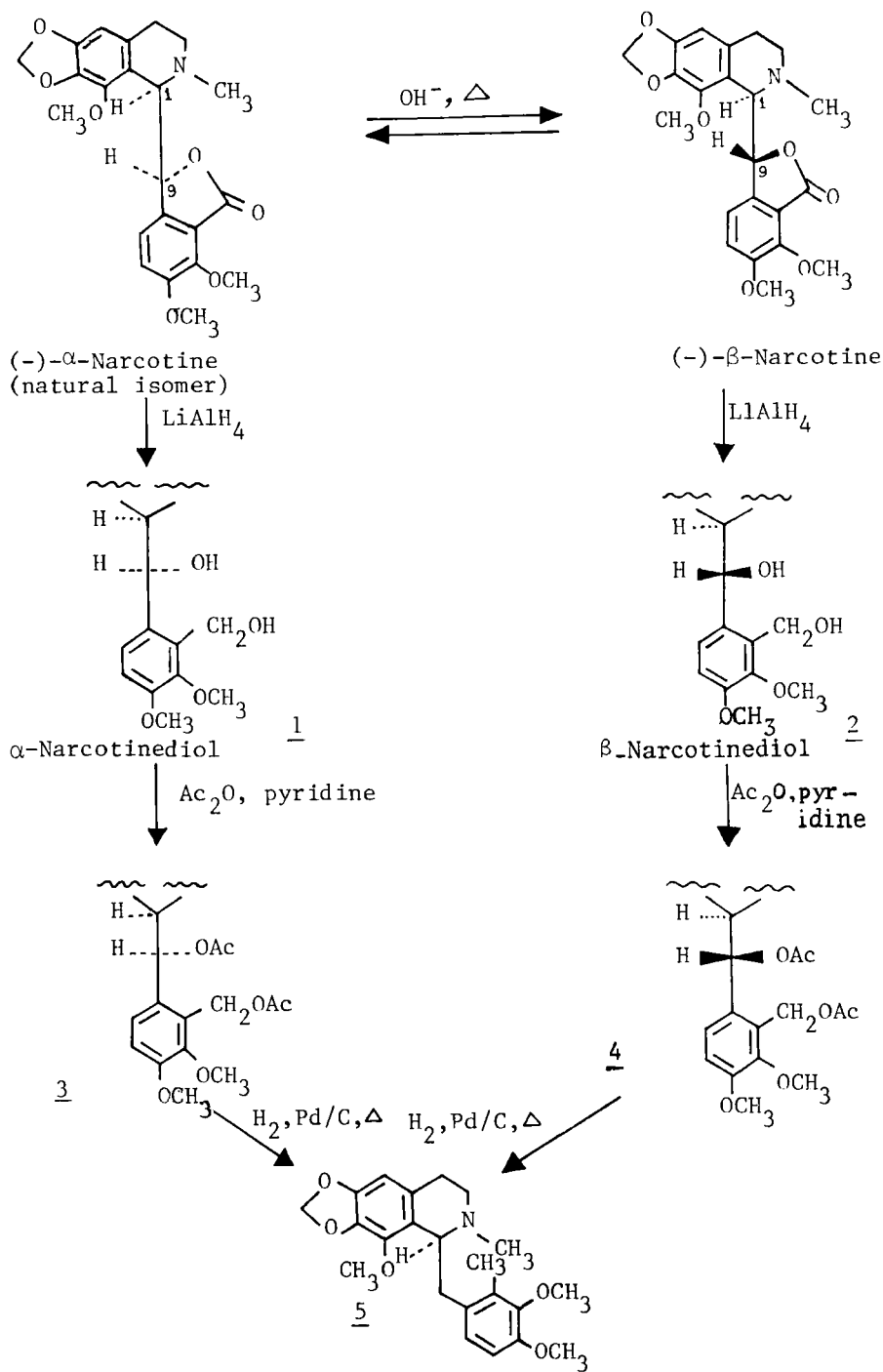
Acetylation of these diols gives rise to the corresponding diacetates 3 and 4, but subsequent catalytic hydrogenolysis yields one and the same dextrorotatory benzylisoquinoline 5. The foregoing sequence clearly establishes that α - and β -noscapine must differ from each other only in their stereochemistry at C-9.

The benzylisoquinoline 5 shows a positive Cotton effect near 295 m μ , so that its C-1 hydrogen must be alpha as indicated. It follows that the C-1 hydrogen in (-)- α -narcotine and in (-)- β -narcotine must also be alpha (Scheme 2).

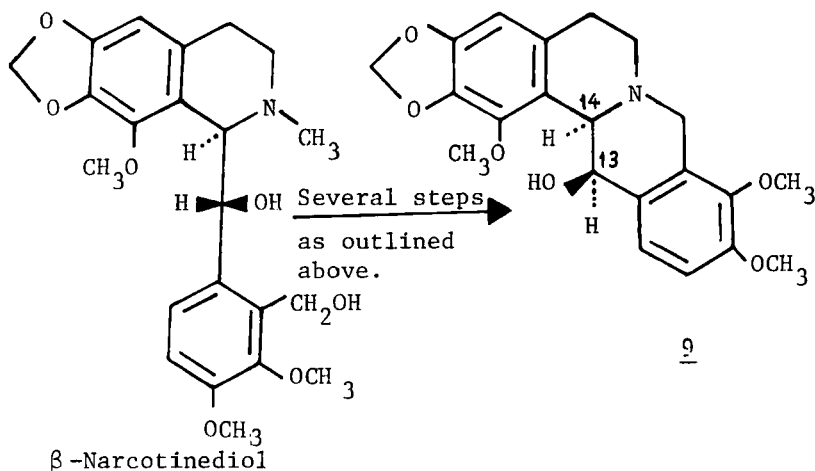
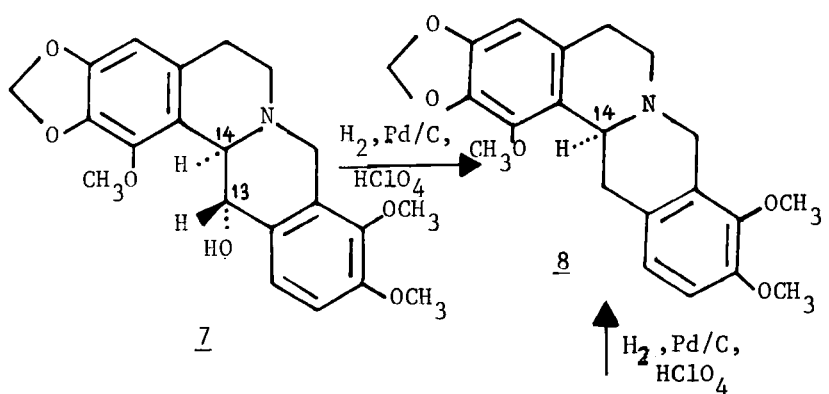
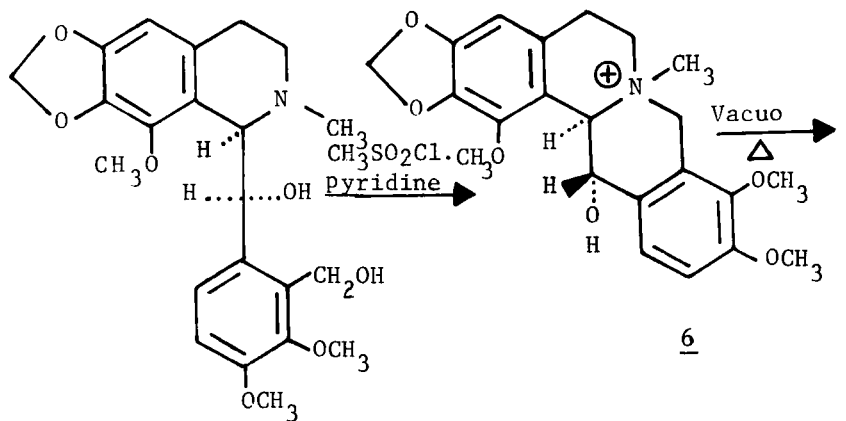
Alternatively, α -narcotinediol was cyclized via its monomesylate derivative to the N-methotetrahydroprotoberberine salt 6. This material underwent N-demethylation on pyrolysis to yield the protoberberine base 7. Reductive removal of the hydroxyl group was achieved in ethanolic perchloric acid over a palladium catalyst. The tetrahydroprotoberberine 8 thus obtained showed a strong negative rotation, so that its C-14 hydrogen must be alpha.

The identical sequence was carried out using β -narcotinediol to yield the tetrahydroprotoberberine base 9. Hydrogenolytic cleavage of this species then provided the same levorotatory tetrahydroprotoberberine 8. The conclusion is that the C-1 hydrogens in both α - and β -noscapine are alpha (Scheme 3).

Turning to the stereochemistry at C-9 for (-)- α - and (-)- β -narcotine, molecular models indicated that the dihedral angle between the protons at C-13 and C-14 of the 13- α -hydroxy



Scheme 2



Scheme 3

base 7 is about 160° . On the other hand, for the 13- β -hydroxy base 9 derived from β -narcotine, this angle is only about 60° . Following exchange of the hydroxylic protons for deuterium, it was determined that the splitting constant $J_{13,14}$ was 9 Hz for species 7, and only about 1.5 Hz for 9. The large coupling value of 9 Hz is in accord with a trans arrangement of the C-13, 14 hydrogens in 7, and the small coupling constant of 1.5 Hz argues for a cis relationship in 9, thus settling the stereochemistry at C-9 for α - and β -noscapine.

1.3 Molecular Weight

413.43

1.4 Elemental Composition

C, 63.91%; H, 5.61%; N, 3.39%; O, 27.09%

1.5 Appearance, Color, Odor and Taste

Noscapine occurs in the form of Orthorhombic bispheroidal prisms, tablets from diacetone or as fine, almost white crystalline powder. Triboluminescent d 1.395. It is odorless and tasteless.

2. Physical Properties

2.1.1 X-ray diffraction

Crystallographic data for noscopine are scarce. The only reported data is due to Lovell (10) and Steward and Player (11). These are as follows:

Long needle-shaped crystals were obtained by recrystallisation of the commercial noscapine from ethanol or methanol. Weissenberg photographs taken with $\text{Cu K}\alpha$ (1.5418 \AA) radiation revealed the following systematic absences:

$$\begin{aligned}h00, h &= 2n + 1 \\0k0, k &= 2n + 1 \\00l, l &= 2n + 1\end{aligned}$$

defining unambiguously the space group $P2_12_12_1$. Cell dimensions were obtained from 2 θ values of 32 reflexions from noscapine using two axes in each case, measured with a counter diffractometer. The following data were obtained:

M.W.	413.41
M.p. ($^{\circ}\text{C}$)	178
Crystal system	Orthorhombic
Space group	$P2_12_12_1$
Cell	} a 15.398(12) b 32.686(36) c (prism) 8.022(8)
Dimensions	
(\AA)	
$V(\text{\AA}^3)$	4037(11)
Z	8
$Q_{\text{calc}}(\text{g}\cdot\text{cm}^{-3})$	1.360
$Q_{\text{exp}}(\text{g}\cdot\text{cm}^{-3})$	1.38

2.1.2 Melting Point

174-176 $^{\circ}\text{C}$ (3)

176 $^{\circ}\text{C}$ sublimes at 150 $^{\circ}\text{C}$ -160 $^{\circ}\text{C}$ under 11 mm pressure at 2 mm distance (1)

2.2 Solubility

It is insoluble in water; slightly soluble in alcohol (95%), in ether and in carbon tetrachloride. Soluble in chloroform, benzene and very soluble in acetone (12).

2.3 Dissociation Constant

It is a very weak base, pK_a 7.8 (1) and 4.85 in 80% methylcellosolve (13).

2.4 Optical Rotation

$[\alpha]_D + 42^{\circ}$ to $+ 48^{\circ}$ (2% w/v in 0.1 M hydrochloric acid) (3)

$[\alpha]_D - 198^{\circ}$ (1% w/v in chloroform),

- $[\alpha]_D$ - 146 (2% w/v in toluene),
 $[\alpha]_D$ - 147⁰ (1.59% in benzene),
 $[\alpha]_D$ + 50 (1% w/v in hydrochloric acid) (14)

2.5 Spectral Properties

2.5.1 Ultraviolet Spectrum

The UV spectrum of noscapine in methanol was scanned from 200 to 400 nm using Varian Carry 119 Spectrophotometer. It exhibits a characteristic UV spectrum (Fig. 1) with two maxima:

λ_{\max}	$E_{1\text{ cm}}^{1\%}$	
310.2	114.9	(C, 9.42 mg per 100 ml)
290.6	106.4	(C, 9.42 mg per 100 ml)

Other UV spectral data of noscapine have also been reported:

λ_{\max}	Log ϵ	
209	4.86)
291	3.60) in ethanol (1, 15)
309-310	3.69)
λ_{\max}	(ϵ)	
291	about 1.1)
310	about 1.4) in alcohol 95% (3)
291	3981)
309	4898) in methanol (12)

2.5.2 Infrared Spectrum

The IR spectra of noscapine as KBr disc and nujol mull were recorded on a Perkin Elmer FT-680B spectrophotometer and shown in Fig.2 & Fig.3 respectively. The structural assignments have been correlated with the following band frequencies (Table 1)

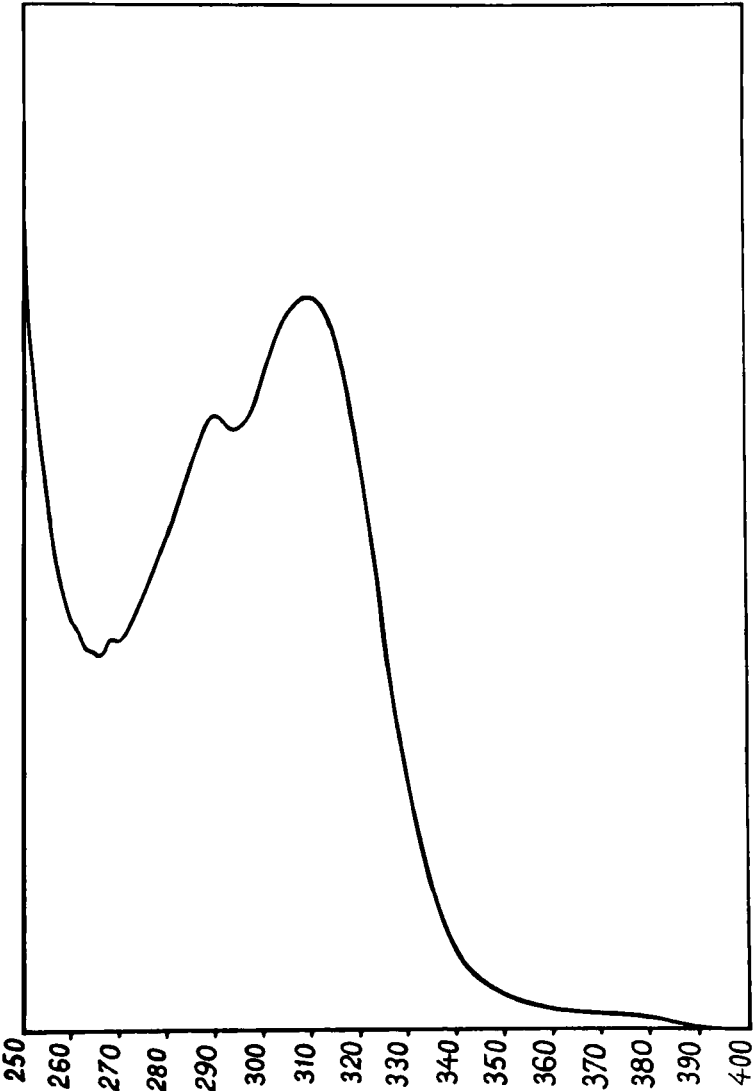


Fig. 1. UV Spectrum of Noscapine in Methanol.

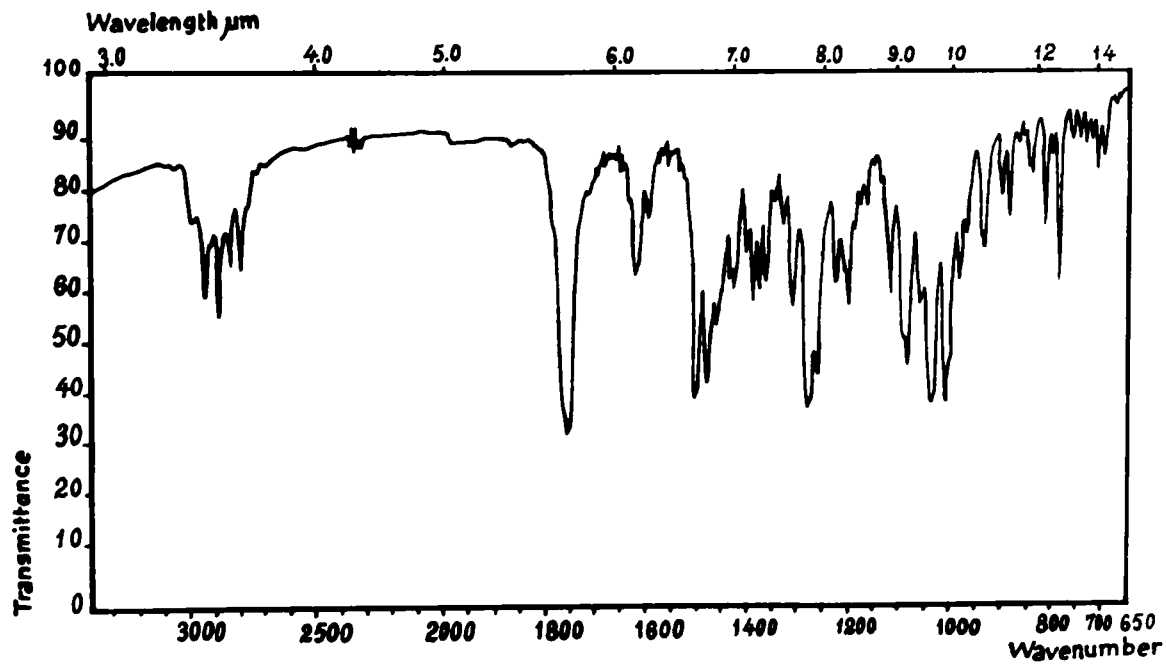


Fig. 2. IR Spectrum of Noscapine as KBr disc.

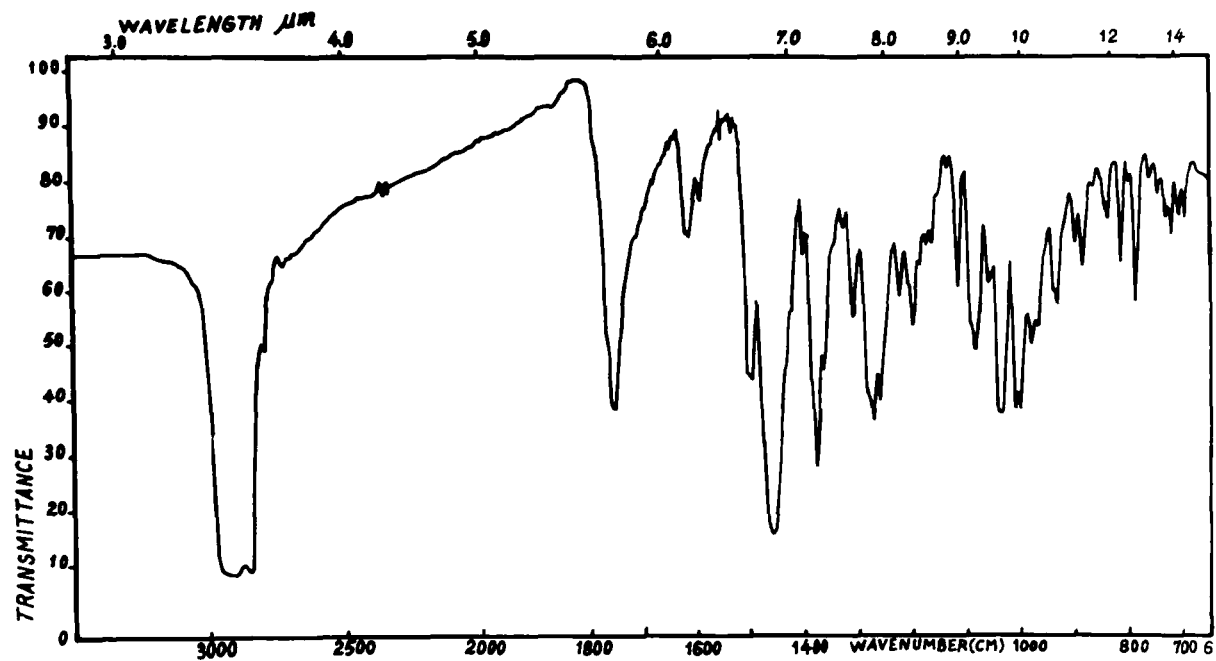


Fig. 3. IR Spectrum of Noscapine as Nujol Mull.

Table 1. IR Characteristics of Noscapine

<u>Frequency cm^{-1}</u>	<u>Assignment</u>
3000, 2945, 2880, 2845, 2800	Methylenedioxy and C-H and $-\text{CH}_3$ frequencies.
1760	(γ -lactone) $3-\text{C}=\text{O}$ group
1625	$-\text{C}=\text{C}-$
1600, 1505, 1480, 1280-1225	Aromatic Aromatic methoxy-aryl C-O stretching vibrations.
790, 815, 835, 885	2 adjacent H atoms, isolated H atom C-H out of plane deformation. Tetra and penta substituted benzenes.

Other characteristic absorption bands are:

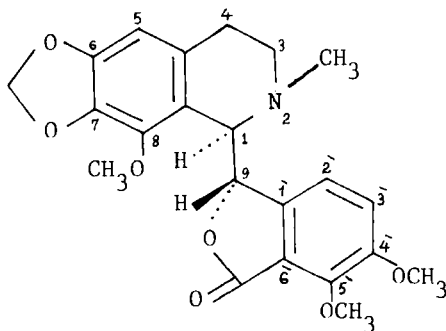
1460, 1430, 1405, 1390, 1380, 1365, 1330, 1310, 1200, 1120, 1085, 1040, 1010, 980, 930, 900, 800, 765, 750, 735, 725, 715, and 700 cm^{-1} .

Other IR data are also reported (16)

2.5.3 Nuclear Magnetic Resonance Spectra

2.5.3.1 Proton Spectrum

The PMR spectrum of noscapine in deuterated chloroform was recorded on a Varian XL200, 200 MHz NMR spectrometer using tetramethylsilane as a reference standard (Fig. 4). The following structural assignment have been made (Table 2).



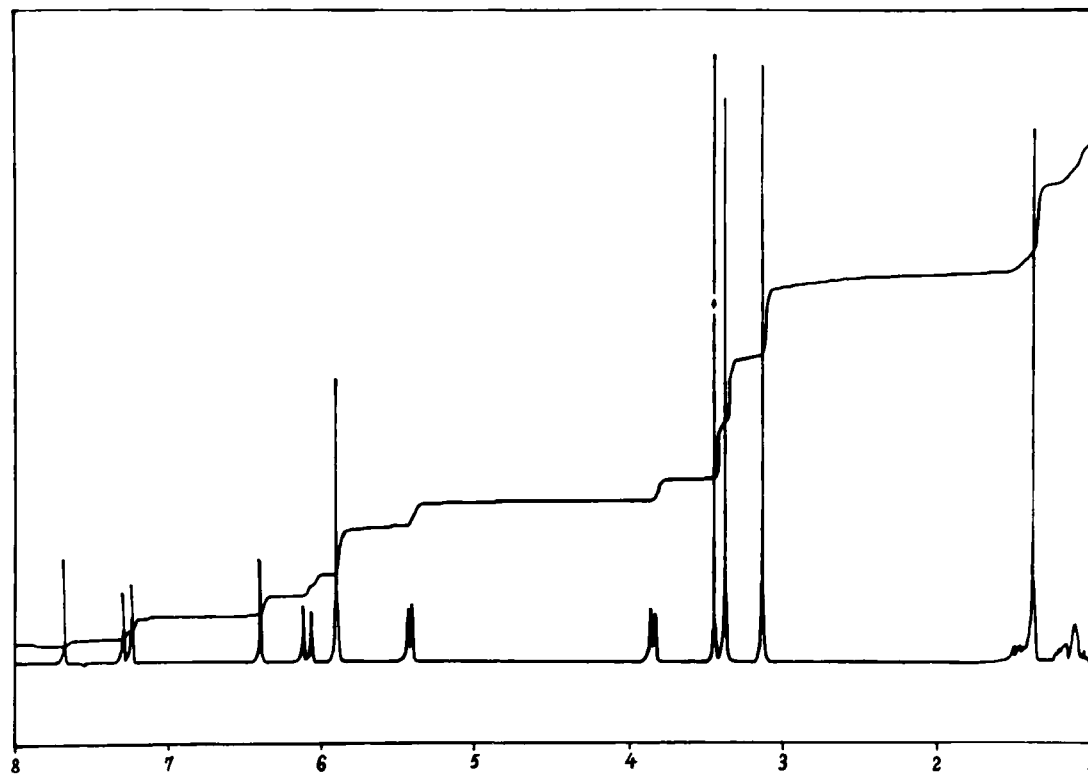


Fig. 4. PMR Spectrum of Noscapine and Tetramethylsilane in Deuterated Chloroform.

Table 2. PMR Characteristics of Noscapine

<u>Assignment (Group)</u>	<u>Position</u>	<u>Chemical Shift (δ)</u>
-CH ₂ -CH ₂	3,4 of isoquinoline	2.32 (m)
N-CH ₃	2 of isoquinoline	2.53 (s)
OCH ₃	8 of isoquinoline	3.84 (s)
OCH ₃	4`of phthalidyl	4.02 (s)
OCH ₃	5`of phthalidyl	4.08 (s)
-CH-	1 of isoquinoline	4.37 (d)
-CH-	9 of phthalidyl	5.55 (d)
-CH ₂ -	methylenedioxy	5.92 (s)
-CH-	2`of phthalidyl	6.05 (d)
-CH-	5 of isoquinoline	6.29 (s)
-CH-	3`of phthalidyl	6.94 (d)

s = singlet, d = doublet, m = multiplet

Other PMR spectral data was also reported (17 and 55).

2.5.3.2 ¹³C-NMR Spectra

¹³C-NMR completely decoupled and off-resonance spectra are shown in Fig. 5 and Fig. 6 respectively. Both were recorded over 11001.1 Hz range, in deuterated chloroform (CDCl₃) on XL-200, 200 MHz NMR spectrometer. Using 10 mm sample tube and tetramethylsilane as reference standard at 25°C.

The carbon chemical shifts assigned on the basis of the additivity principals and off-resonance splitting pattern (Table 3) (18).

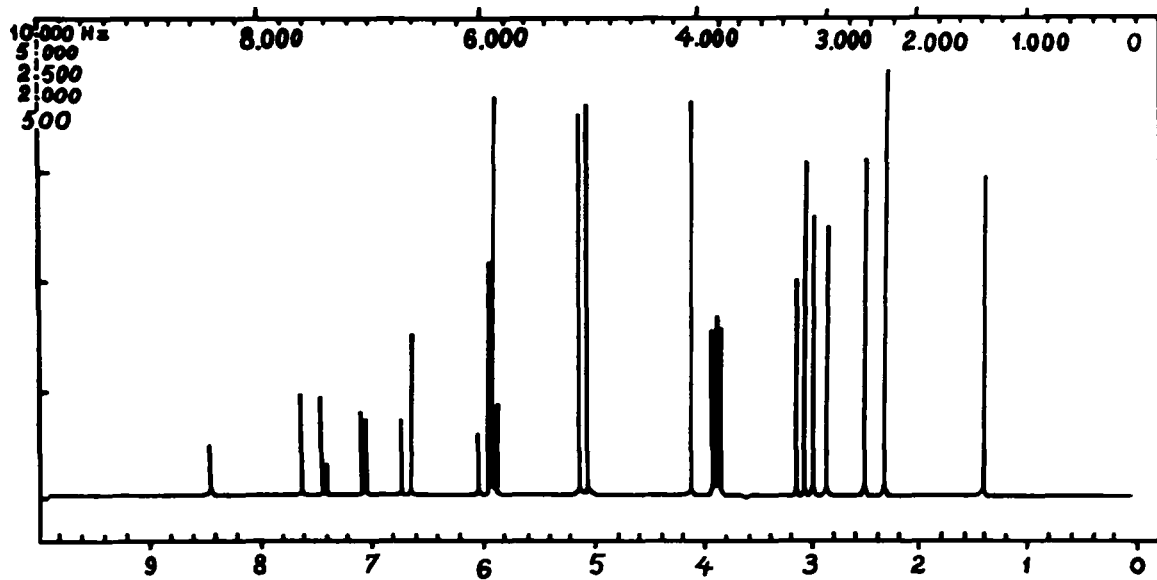


Fig. 5. ^{13}C -NMR Spectrum of Noscapine in Deuterated Chloroform.

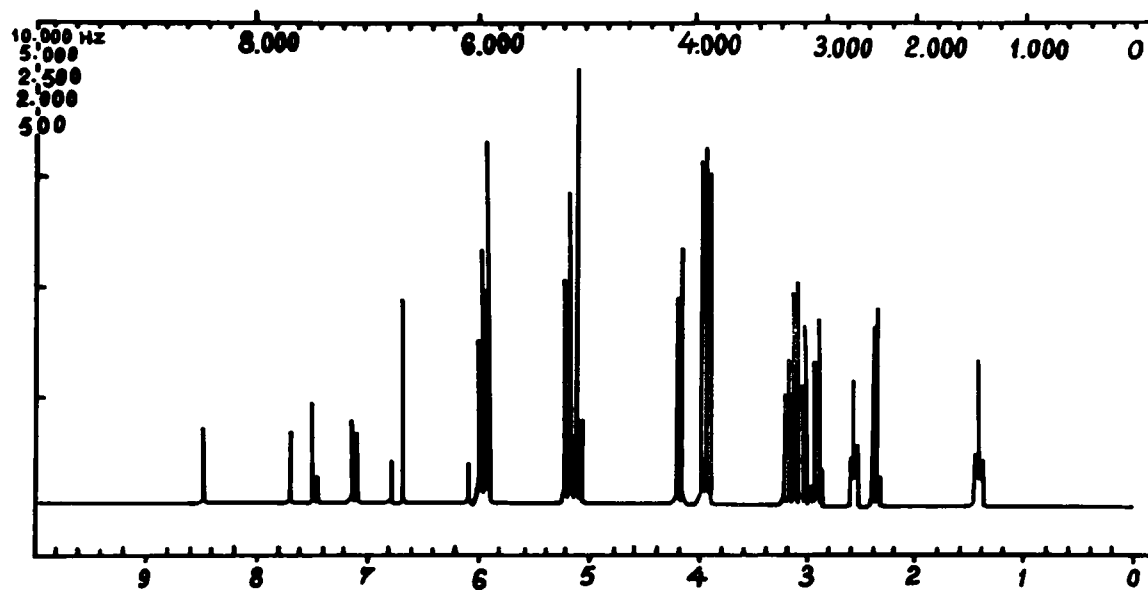


Fig. 6. ^{13}C -NMR Off-Resonance Spectrum of Noscapine in Deuterated Chloroform.

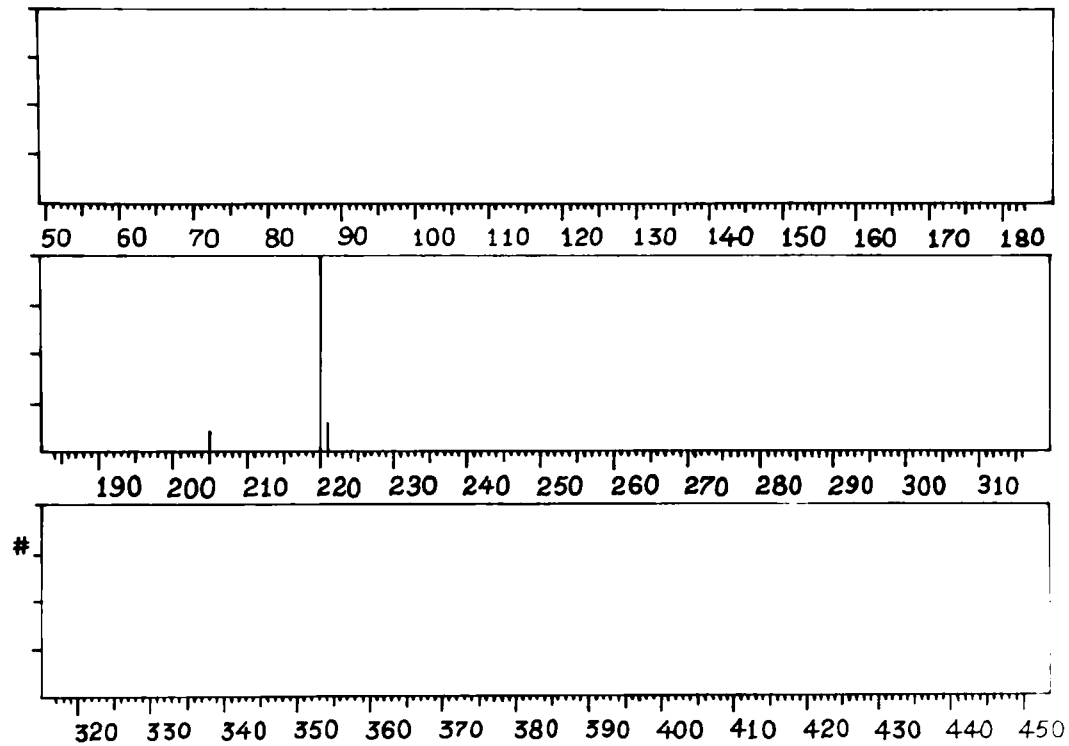


Fig. 7. EI-Mass Spectrum of Noscapine.

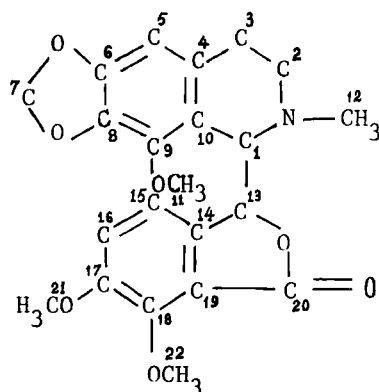


Table 3. Carbon Chemical Shifts of Noscapine

Carbon No.	Chemical Shift ppm	Carbon No.	Chemical Shift ppm
C-1	60.84 (d)	C-12	46.29 (q)
C-2	49.99 (t)	C-13	81.83 (d)
C-3	28.03 (t)	C-14	120.17 (s)
C-4	134.03 (s)	C-15	118.19 (d)
C-5	117.65 (d)	C-16	102.29 (d)
C-6	140.45 (s)	C-17	147.67 (s)
C-7	100.73 (t)	C-18	148.37 (s)
C-8	141.14 (s)	C-19	117.11 (s)
C-9	152.18 (s)	C-20	168.06 (s)
C-10	132.09 (s)	C-21	56.78 (q)
C-11	59.36 (q)	C-22	62.22 (q)

2.5.4 Mass Spectrum

The mass spectrum of noscapine obtained by electron impact ionization and which was recorded on Ribermag R-10-10 mass spectrometer equipped with direct inlet probe. The spectrum (Fig. 7) shows molecular ion peak and shows a base peak at m/e 220.

The mass spectrum of noscapine obtained by butane chemical ionization (Fig. 8) shows a molecular ion peak M^+ at m/e 413 with a relative intensity of 2.8% and a base peak at m/e 220. The most prominent fragments,

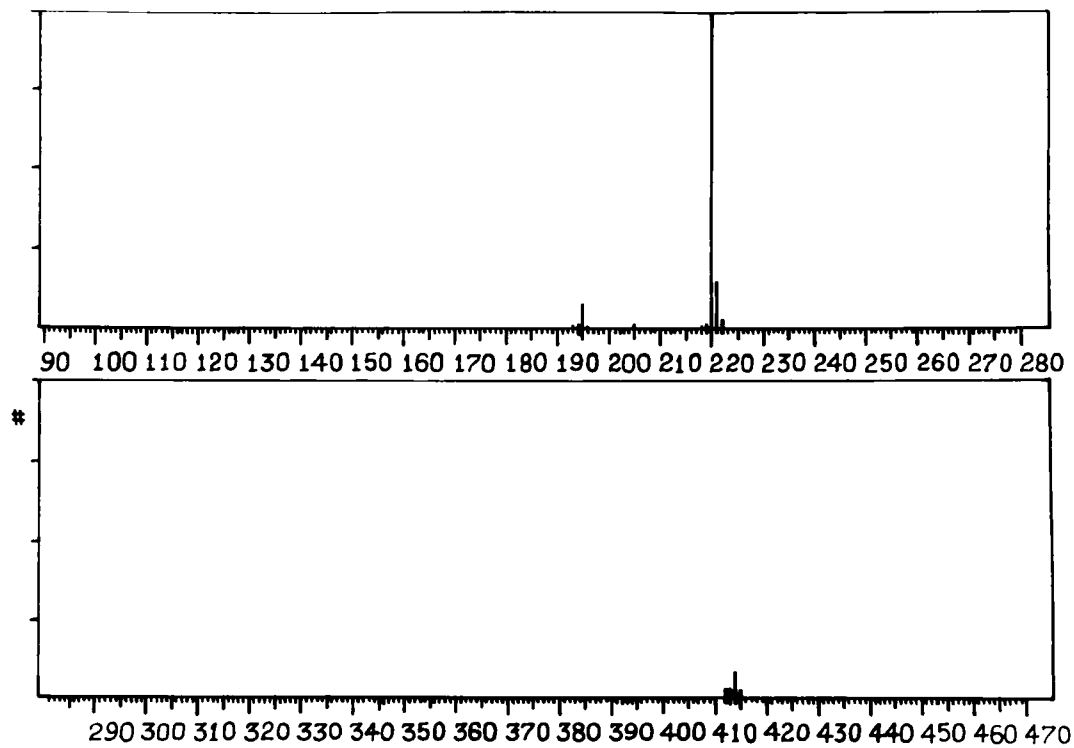
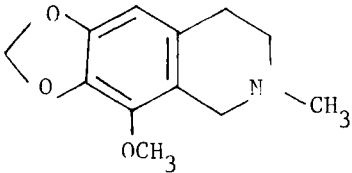
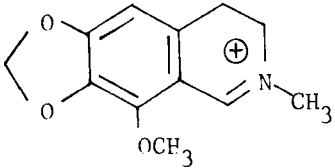
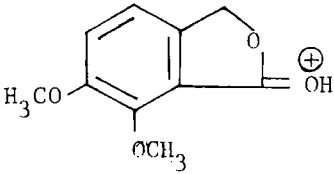
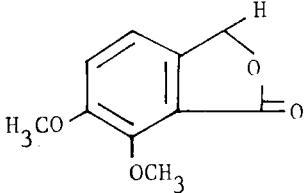


Fig. 8. CI-Mass Spectrum of Noscapine.

their relative intensities and structures are listed in Table 4.

Other mass spectral data for phthalideisoquinolines was also reported (19, 20).

Table 4. Mass Fragments of Noscapine

<u>m/e</u>	<u>Relative Intensity %</u>	<u>Fragment</u>
413	2.8	M^+
221	15.0	
220	100.0	
195	8.0	
193	0.9	

3. Preparation

3.1 Isolation from Opium

Noscapine occurs up to 11% naturally in opium (Papaver somniferum L. (Fam. papaveraceae). It was first discovered by Derosne in 1803 (20), and isolated by Robinquet in 1817 (21). Noscapine can be separated from other opium alkaloids by the procedure outlined in Scheme 4 (90).

Another patent method has been also described for its isolation on an industrial scale (22).

4. Synthesis of Noscapine

4.1 By Tissue Culture Method

Khanna et al (23) described a method for the synthesis of noscapine along with other alkaloids by tissue culture of Papaver somniferum Linn.

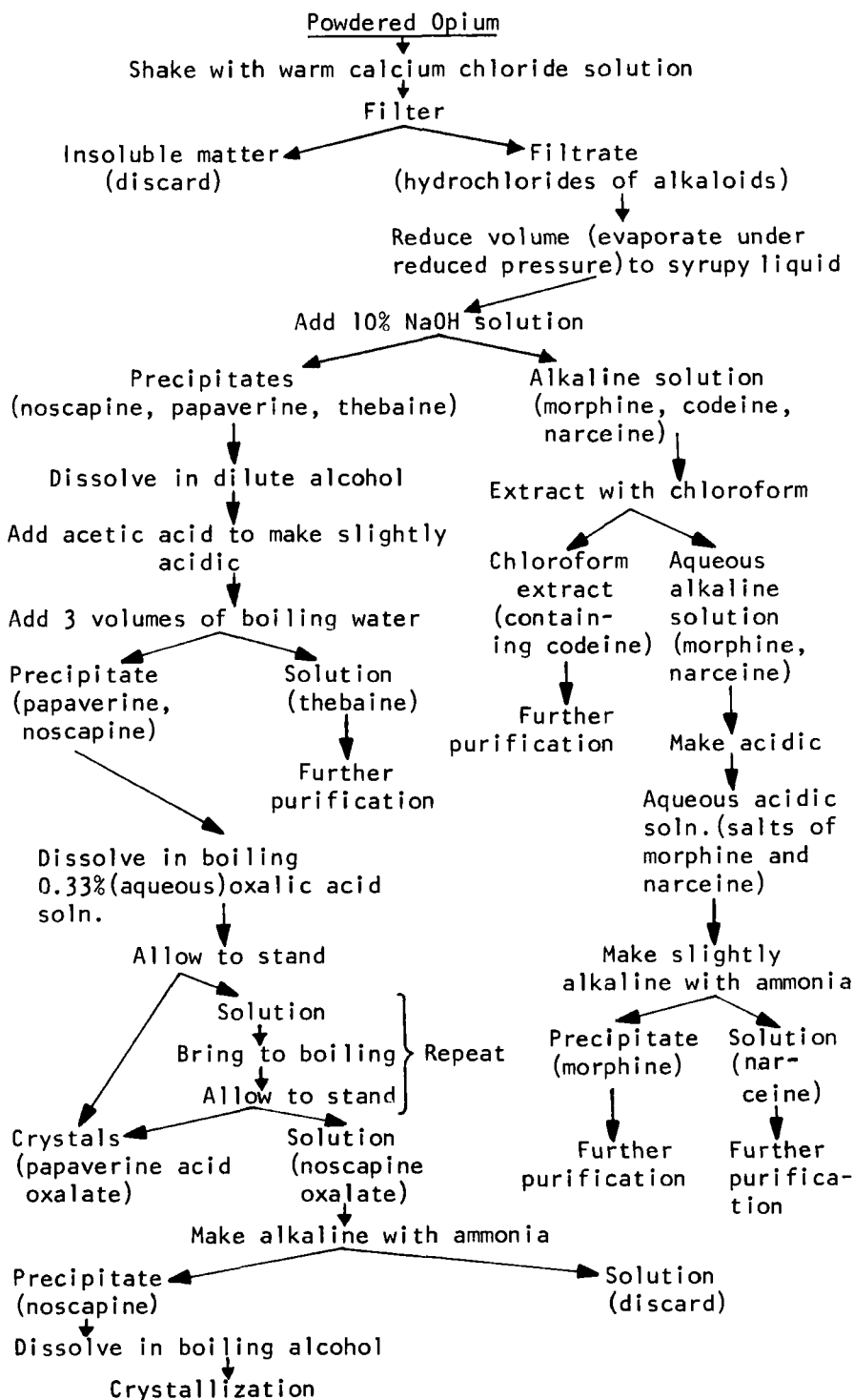
4.2 By Chemical Methods

Perkin and Robinson (24) discovered that heating a mixture of cotarnine 1 and meconine 2 in ethanol resulted in a small yield of noscapine 3. The expected second isomer of noscapine (because of the presence of 2 assymetric centres) was not found. The synthetic noscapine was then resolved and the noscapine obtained shown to be identical with the natural product. (Scheme 5).

A quite efficient synthesis of noscapine was developed by Hope and Robinson in 1914 (25), in which cotarnine 1 is condensed with iodomeconine 2 and the adduct was reduced with sodium amalgum to give the desired product, corresponding to the natural series (Scheme 6).

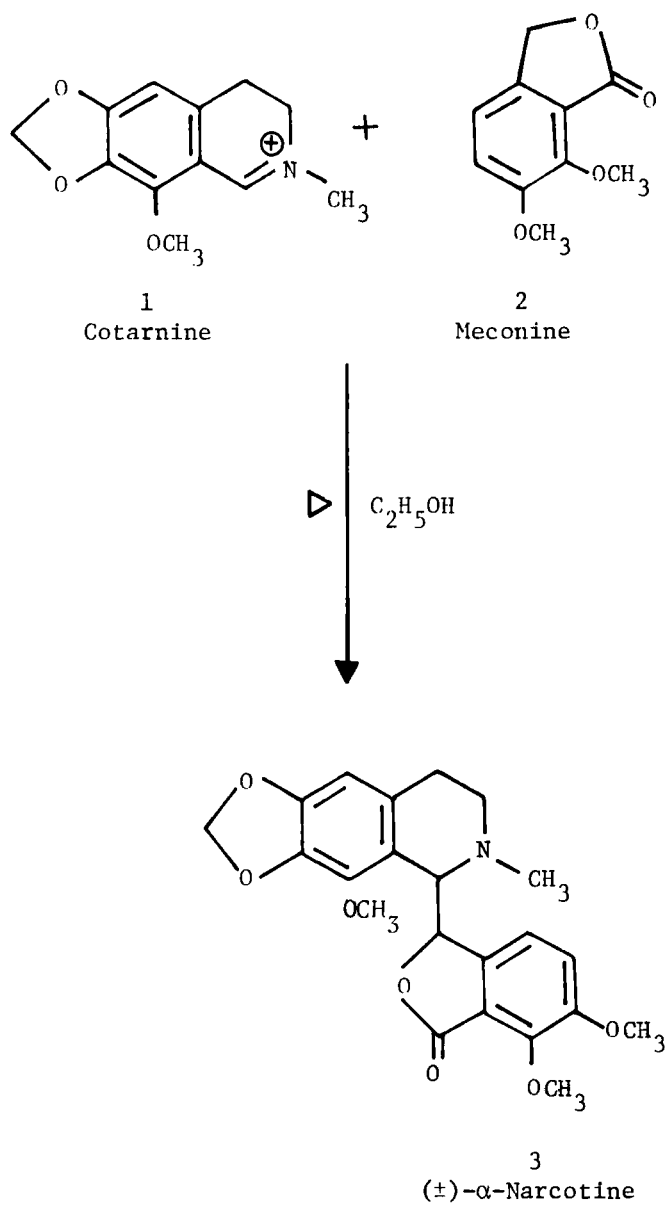
5. Biosynthesis of Noscapine

It has been postulated that the phthalideisoquinolines are formed in nature by oxidative modification of tetra-hydroprotoberberines, and previous work with labeled precursors supports this hypothesis (26).

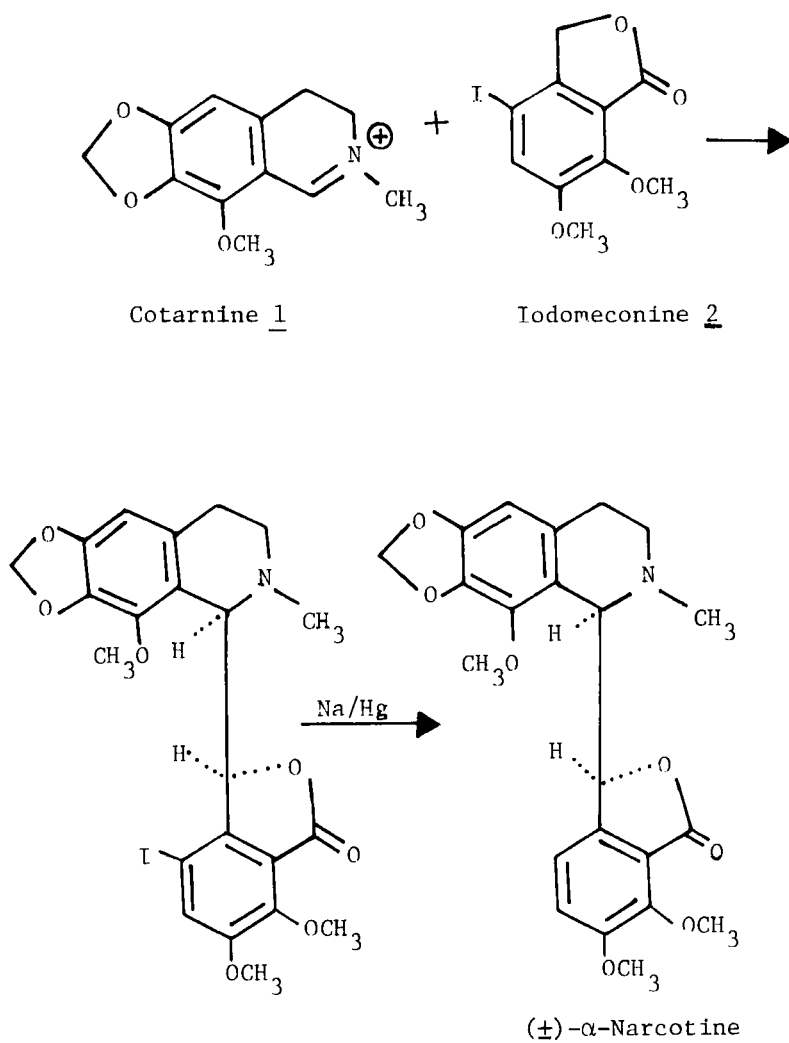


Scheme 4: Isolation of

from powdered opium.



Scheme 5



Scheme 6

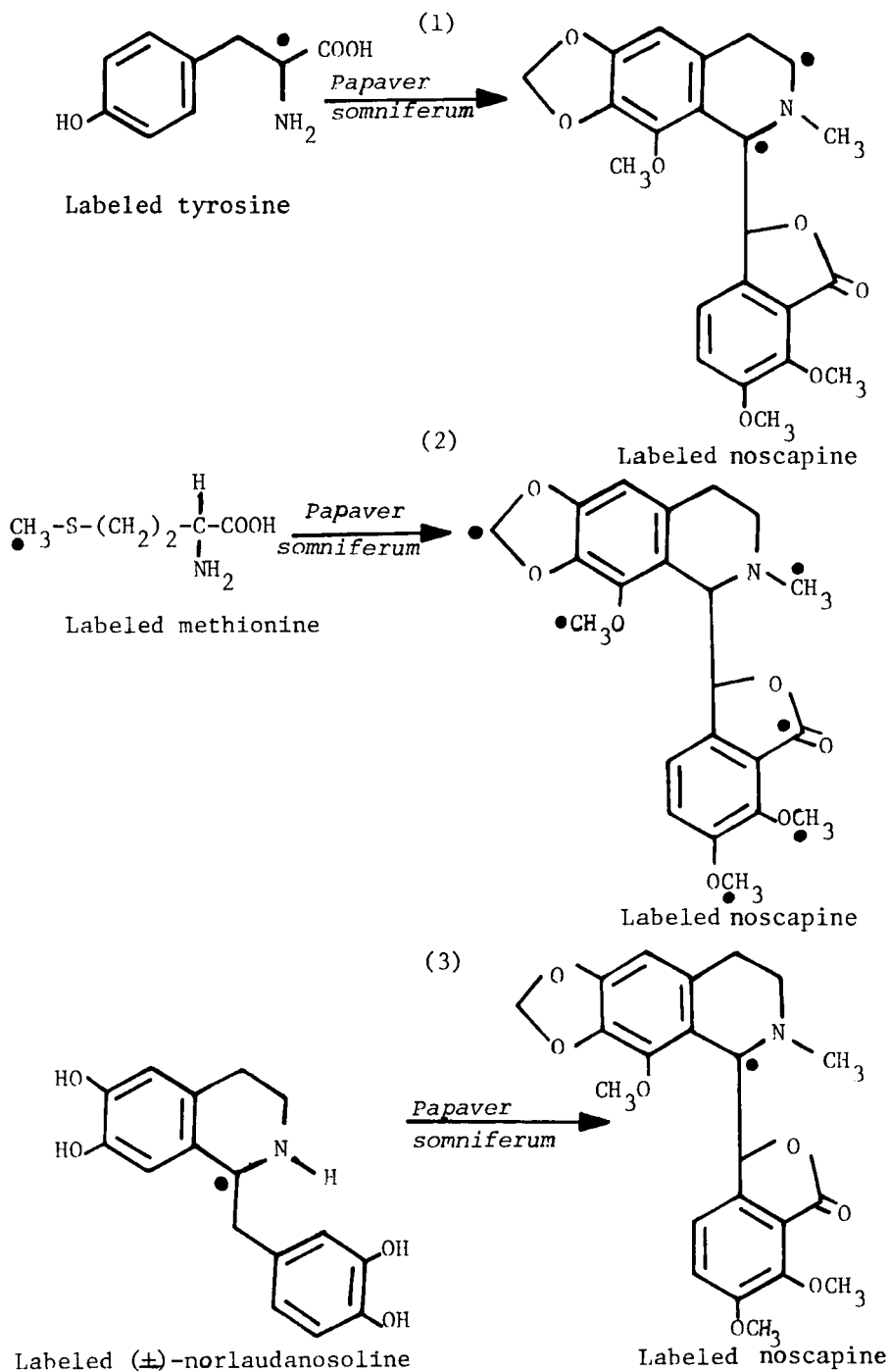
Several feeding experiments (6,8, 94) have been run to elucidate the biogenesis of noscapine in Papaver somniferum L. (Papaveraceae). When labeled (\pm)-tyrosine was fed to the plant, radioactive narcotine labeled specifically and equally at C-1 and C-3 was obtained. The benzylisoquinoline system of noscapine is thus derived biologically from two Ar-C-C units which can arise from tyrosine.

The carbon atoms that arise from the S-methyl of methionine were clearly pinpointed when, after feeding radioactive methionine, noscapine labeled at the lactone carbonyl, the methylenedioxy group, and the N- and O-methyl carbon atoms was obtained (27, 28). Progressing further along the biogenetic locus, the benzylisoquinoline (\pm)-norlaudanoline labeled C-1 led to noscapine also labeled C-1 (29). Even more significantly, when quadruply labeled (+)- and (-)-reticuline were fed separately to P. somniferum, it was found that both enantiomers were incorporated into noscapine, but with the (+)-isomer doing so slightly more efficiently. Evidently epimerization of the wrong benzylisoquinoline precursor must occur, probably by oxidation-reduction at C-1. In keeping with this conclusion considerable loss of tritium occurred in the course of incorporation of both reticulines. Another important observation is that the lactone carbonyl of the phthalideisoquinoline must be derived from the N-methyl group of the benzylisoquinoline precursor (27, 29, 30).

Finally, it has been found that the feeding of labeled (-)-scoulerine results in the formation of radioactive noscapine. Protoberberines are, therefore, the precursors for the phthalideisoquinolines in plants. Significantly, (-)-scoulerine, which possesses the same absolute configuration as (+)-reticuline and (-)- α -narcotine, was more than one hundred times more efficient than its enantiomer as a precursor for (-)- α -narcotine. The biogenetic sequence in plants is, therefore, benzylisoquinolines \rightarrow tetrahydroprotoberberines \rightarrow phthalideisoquinolines. The biosynthesis of (-)- α -narcotine is shown in Scheme 7.

6. Metabolism

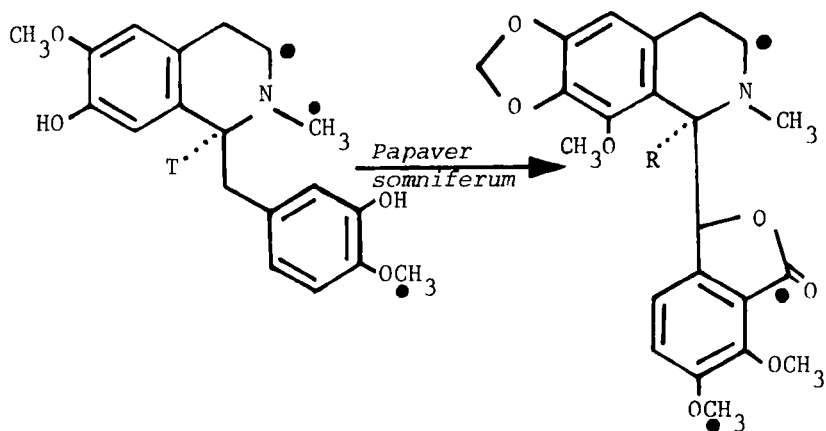
The metabolism of noscapine was reported



Scheme 7

Scheme 7 (continued)

(4)



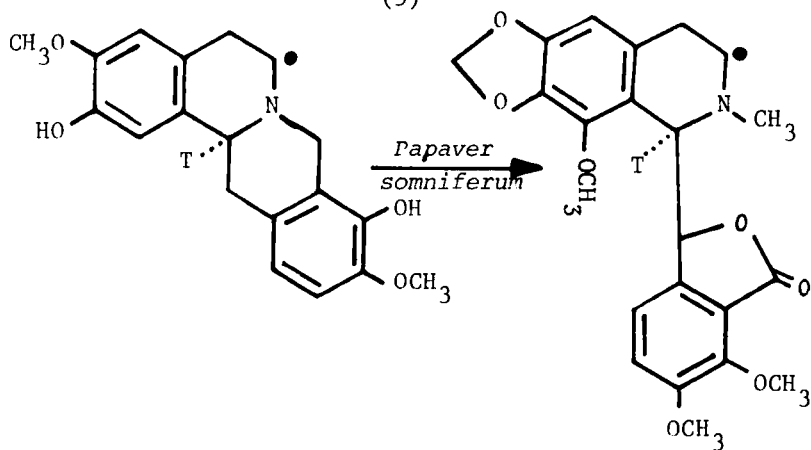
Labeled (+)-reticuline

(Labeled (-)-reticuline somewhat
less efficient)

R = H or T

Labeled noscapine
(appreciable loss of
tritium)

(5)



Labeled (-)-scoulerine

Labeled noscapine
(Some tritium loss)

(31, 32). Oral administration of _____ to male rabbits and examination of the 24 hours urine by preparative TLC and methane chemical ionization mass spectrometry revealed the presence of two O-monodemethylated compounds as free metabolites 2 and 6, one O-didemethylated derivative 3 or 4 and their conjugated forms. Noscaphine given orally to rats was metabolised to di-O-demethyl-noscaphine 3 or 4, cotarnine 5, hydrocotarnine 7, oxycotarnine 9, and O-demethylmeconine 6. These metabolites were isolated from urine.

All possible metabolites of noscaphine are shown in Scheme 8.

7. Methods of Analysis

7.1 Identification Tests

The following identification tests are described by the British Pharmacopoeia (1980).

- a) The light absorption, in the range 230 to 350 nm, of a 0.005 per cent w/v solution in methanol exhibits two maxima, at 291 nm and 310 nm and a minimum at 263 nm; ratio of the absorbance at the maximum at 310 nm to that at the maximum at 291 nm, about 1.2.
- b) To 10 mg add 0.5 ml of sulfuric acid and mix; a greenish-yellow solution is formed which turns red and finally violet on heating.
- c) Solutions in organic solvents, such as methanol and chloroform, are levorotatory; aqueous acidic solutions are dextrorotatory.

7.2 Microcrystal Tests

- a) According to the method of Clarke and Williams (33), in potassium chromate solution, noscaphine forms feathery rosettes or bunches of blades, sensitivity being 1 in 1500 (Fig. 9).
- b) In sodium carbonate solution rosettes and bunches of needles are seen at the same sensitivity (Fig. 10).

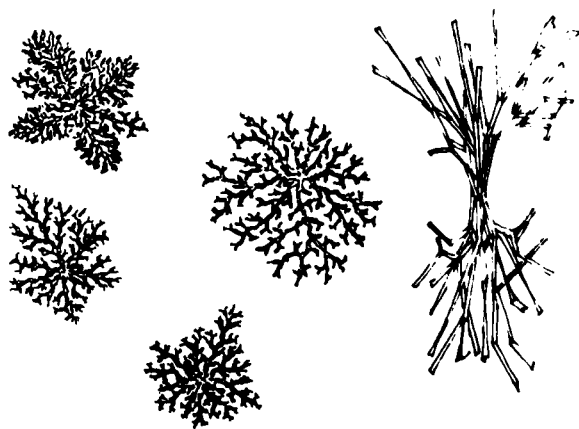


Fig. 9. Crystals of Noscapine with Potassium Chromate Solution.

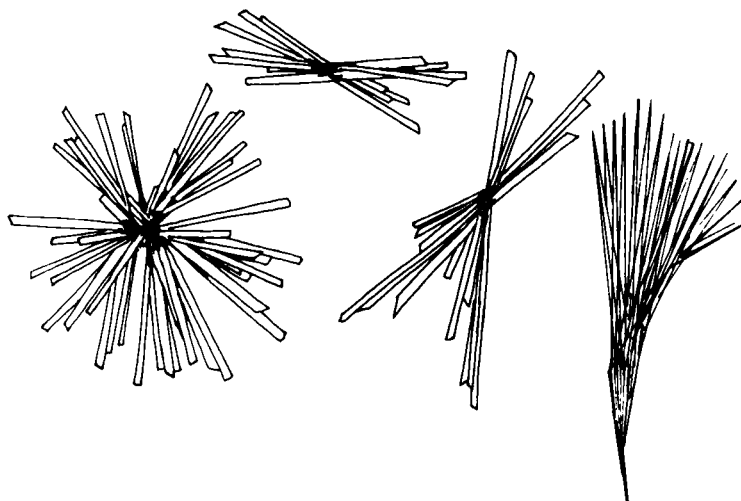
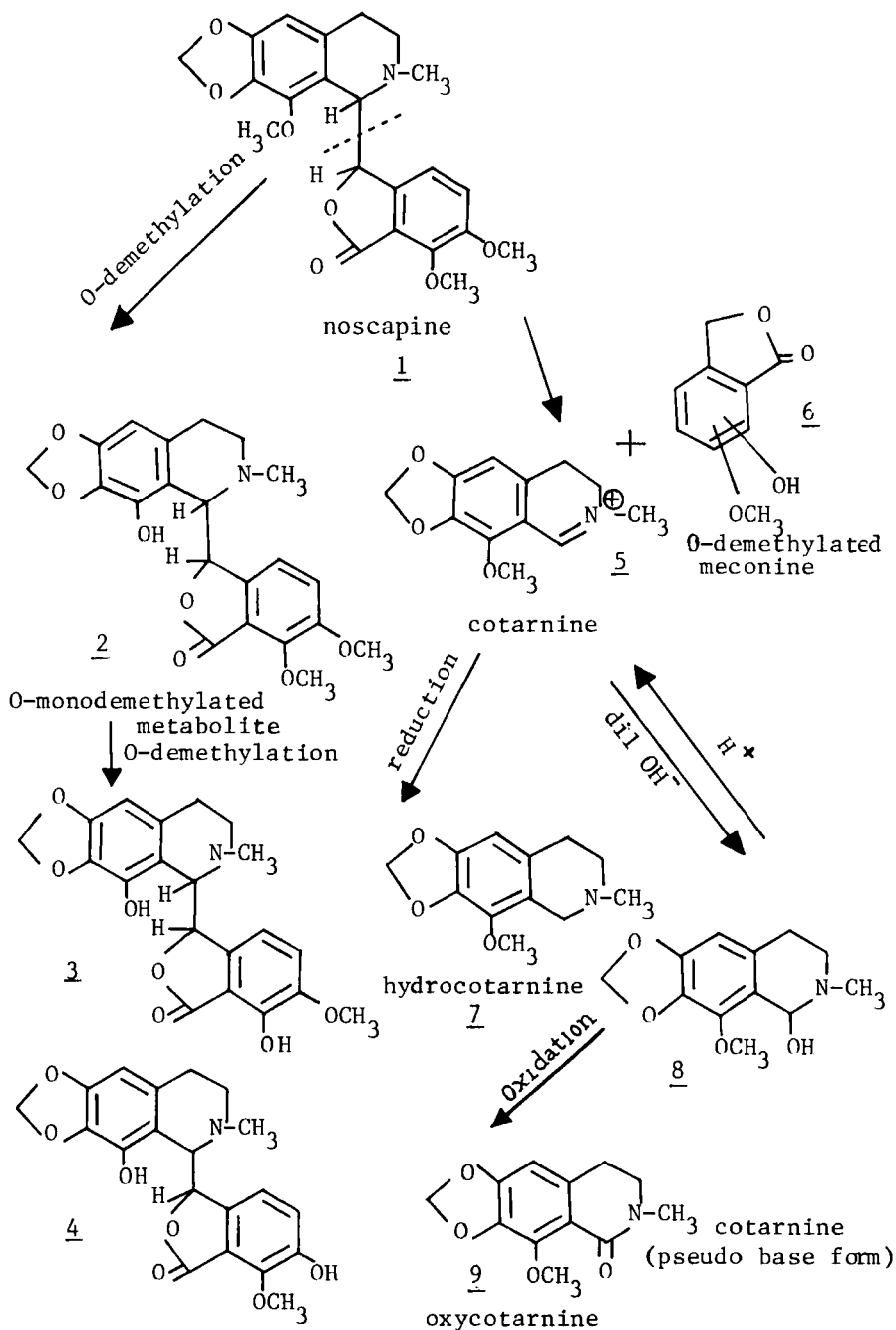


Fig. 10. Crystals of Noscapine with Sodium Carbonate Solution.

Scheme 8. Possible Metabolites of (-)- α -narcotine

O-didemethylated metabolites

7.3 Titrimetric Methods

The official methods of determining Noscapine are described by the B.P. (3) and U.S.P. (34).

7.3.1 Non-Aqueous Titration

The B.P. (3) describes the following method:-

Dissolve 0.5 g in 40 ml of anhydrous glacial acetic acid previously neutralised to crystal violet, warming gently. Titrate with 0.1 M perchloric acid using 0.25 ml of crystal violet solution as indicator. Each ml of 0.1 M perchloric acid is equivalent to 0.04134 g of $C_{22}H_{23}NO_7$.

The U.S.P. describes the following method:-

Dissolve about 1.5 g of Noscapine, accurately weighed, in 25 ml of glacial acetic acid. Add 25 ml of dioxane and 5 drops of crystal violet T.S., and titrate with 0.1 N perchloric acid in glacial acetic acid to the end-point change from purple to blue. Perform a blank determination, and make any necessary correction. Each ml of 0.1 N perchloric acid is equivalent to 41.34 mg of $C_{22}H_{23}NO_7$.

Another method was described by Tuthill et al. (35) using malachite green as better indicator than crystal violet.

7.3.2 Polarographic Titration

- a) Using a dropping mercury electrode as indicator noscapine hydrochloride can be titrated with Cadmium Iodide in solution of neutral salts (0.1 M KNO_3 , NaCl or Na_2SO_4) (36).
- b) Dusinsky (37) described a method where noscapine can be titrated in alkaline

solution (1.25 N NaOH) showing depression in the polarographic curve at 1.5V.

- c) Sodium alizarinsulphonate has been used for the determination of noscaphine (38). A potential of - 0.65 V was used and the titration medium was 0.3 N KCl adjusted to a pH of 4-6.
- d) Souček and Šýka (39) stated a method using tungstosilicic acid. This acid was found especially useful since the reaction is very sensitive and precipitation is immediate. The polarography also gave the composition of the tungstosilicic acid organic base complex. The poor selectivity of Tungstosilicic acid is due to its high sensitivity which allows accurate determination of 10-20 mg of base. A 0.01 M aqueous solution of tungstosilicic acid is used with a dropping mercury cathode and S.C.E. anode at 0.65 V. The pH of solution adjusted with HCl (0.1 to 0.6 N). Plot of current vs acid used consists of two straight lines and the intersect considered as the equivalence point.
- e) Another method for polarographic titration was also reported (40). This method is based on the formation of complex mercury compounds. Solution of K_2HgI_4 containing an excess of iodide is the most suitable for the determination. The titration is carried out with a dropping mercury electrode at the potential -0.8 V to - 0.9 V (VS. the S.C.E.) with 0.1 M KNO_3 or 0.1 M H_2SO_4 as supporting electrolyte.

7.3.3 Potentiometric Titration

Tungsten rod was used as indicator electrode in the potentiometric titration of noscaphine in a 1:6 mixture of acetic acid: acetic anhydride (41).

7.4 Complexometric

Noscapine is precipitated from 0.5 N HCl with 0.028 M Bi-EDTA and 0.112 M KI forming iodobismuthate complexes and EDTA is being set free (42). After centrifugation the free EDTA is determined in an aliquot of the supernatant liquid with 0.01 M ZnSO₄ in pH 9.1 borate buffer and Eriochrome black T as indicator. Tertiary amines, quaternary ammonium salts or analogous sulphonium, phosphonium and arsonium compounds interfere in the determination.

7.5 Spectrophotometric

7.5.1 Colorimetric

- a) Yoichi and Sano (43) describe a method for analysis of noscapine in mixed pharmaceutical preparations. The sample is mixed with a solution of chromotropic acid 0.2% in 70% (v/v) H₃PO₄ acid and heated at 100°C for 30 minutes and the extinction is measured at 570 nm. A calibration curve is rectilinear for 30 to 150 µg of noscapine hydrochloride per ml.
- b) Solochrome Green V 150 (C.I. Mordant Green 15) has been used as aqueous 1 mM solution. The complex formed by noscapine is extracted into chloroform and the absorbance is measured at 520 nm (44).
- c) Another method for the quantitative separation of papaverine from noscapine in mixtures was also reported (45). This method is based on the formation of an insoluble papaverine reineckate in acid solution in the presence of excess chloroform.

Procedure

Triturate the sample (4.5 g) with glacial acetic acid (25 ml) followed by H₂O (20 ml) and filter. Extract a 10 ml aliquot with

CHCl_3 (8 X 10 ml) and wash each extract in turn with H_2O (15 ml), H_2O (15 ml) plus NaOH soln. (1 : 1) containing a little NaHSO_3 (15 ml), H_2O (15 ml), 0.1 N H_2SO_4 (15 ml and 10 ml) and 0.05% NaHCO_3 soln. (10 ml). Evaporate the combined washed extracts to dryness on a water bath. Dissolve the residue in CCl_4 (50 ml), strain through cotton-wool and pass through a column of Ca(OH)_2 . Wash the column with CCl_4 (2 X 10 ml) and extract the combined CCl_4 fractions with 0.1 N HCl (2 X 10 ml). Shake the HCl soln. with CHCl_3 (10 ml) for 10 min., add 2% ammonium reineckate soln. (10 ml), shake for 30 min. and filter through sintered glass. To determine papaverine dissolve the ppt. in acetone and measure the extinction at 525 m μ . To determine noscapine, shake the CHCl_3 layer of the filtrate with 0.25% AgNO_3 soln. (40 ml), separate and further extract with CHCl_3 (2 X 10 ml); strain the combined CHCl_3 fractions through cotton-wool, dilute to 250 ml, and either measure the extinction at 310 m μ or evaporate and titrate with 0.05 N HClO_4 in glacial acetic acid.

- d) Thomas described a method for determination of some drugs containing a tertiary-amine group (46). The drug is heated with 10% malonic acid in acetic anhydride at 80° for 15 min. and, after dilution with ethanol, the extinction is measured at 333 nm. the limit of detection for noscapine hydrochloride was 10 to 30 ng ml⁻¹. Dosage forms require preliminary extraction of the drug.

7.5.2 Infra-red

Bakre et al (47) described an infra-red spectroscopic method for the determination of the origin of opium as well as a simultaneous assay of noscapine, thebaine and papaverine. 4.5 g finely ground sample was titrated for 20 min. in 25 ml water was slowly added with continuous stirring and the

resulting solution was filtered. 10 ml Aliquot of filtrate was extracted four times into 10 ml chloroform and each extract was washed with 10 ml water, 25 ml of 0.2% sodium bisulphite in 30% aqueous sodium hydroxide, 10 ml water and again 10 ml water. The combined chloroform solution was filtered through cotton wool and evaporated. The residue is dried in a desiccator then mixed with anhydrous carbon tetrachloride, filtered through sintered glass and diluted to 25 ml. The IR is examined from 1100 cm^{-1} to 1900 cm^{-1} in a 1 mm sodium chloride cell, noscapine being measured at 1767 cm^{-1} . The absorbance is compared with absorbances of solutions of known concentrations.

Other IR methods for determination of isoquinoline alkaloids were also reported (48, 49).

7.5.3 Ultra-Violet

Tetrapon a mixture of the hydrochlorides of Morphine, Noscapine, codeine and papaverine was analysed by Jensen (50). Morphine was separated by extraction with chloroform from strong alkaline solution and determined spectrophotometrically with NaNO_2 at 440 nm. The other alkaloids were separated by T L C on Kieselgel CF 254 with ethanol: benzene 1:4 as solvent. The spots (located in U.V. radiation) were extracted with methanol and determined at 215 nm for codeine, at 279 nm for papaverine and 312 nm for noscapine.

7.5.4 Atomic Absorption

An indirect method for the analysis of Noscapine in drugs was reported (51). A complex is formed between Noscapine and Reinecke salt in the presence of tartaric acid at pH 1.7. This is extracted into chloroform and Noscapine is determined indirectly by measuring chromium cation by atomic absorption spectrophotometry.

7.5.5 Spectrofluorimetric

- a) Noscapine has been determined in mixtures of opium alkaloids (52) by measuring the fluorescence at 375 nm (excitation at 315 nm). The sample is buffered at pH 9 in 0.1 N sulphuric acid and 0.1 N sodium hydroxide. Noscapine is extracted in chloroform and a portion of this extract is treated with trichloroacetic acid in chloroform to quench the fluorescence of papaverine. A standard solution of 2-aminopyridine in 0.1 N sulphuric acid is also measured at 375 nm (excitation at 315 nm). For the calculation each fluorescence reading on the test solution is calculated as a percentage of that for the standard and referred to calibration graphs prepared similarly for Noscapine. Sub-microgram amounts of Noscapine can be determined without preliminary separation.
- b) Vedso stated a method (53) for the determination of Noscapine in plasma and urine. It involves extraction of 1 ml sample at pH 10 into ethyl ether and re-extraction with dilute HCl. The acid is neutralised and the solution was adjusted to PH 9.2 with a borax buffer. Fluorescence is measured through 480 to 580 mμ filter (excitation at 365 mμ) before and after autoclaving at 120°C for 30 min. Standards (0 to 2.5 μg cm⁻³) are also measured in the same way. It was stated that concentrations from 0.05 μg per ml can be determined and although the presence of morphine gave an increase in fluorescence about half that for noscapine, codeine, narceine and papaverine did not interfere.
- c) A reaction mixture of 10% malonic acid in acetic anhydride was used in a method reported by Rao and Tandon (54). Interference was caused by Tertiary amines, glucose, magnesium acetate and some inorganic salts, but not by diethylamine, aniline, benzoic acid, aspirin and saccharin.

7.5.6 Nuclear Magnetic Resonance

A known amount of t-butyl alcohol was added as a standard to noscapine in ethanol free-chloroform and the peaks at 3.83, 4.00 and 4.05 ppm; corresponding to the nine methoxy group protons of noscapine were integrated along with the peak at 1.3 ppm corresponding to the nine methyl-group protons of t-butyl alcohol (Fig. 11). The amount of noscapine is calculated from the integration ratio and the known amount of standard (55).

7.5.7 Mass

Noscapine was identified in opium (92) without any prior separation. Samples are introduced directly into the ion source using a solid sampling probe. Reagent gases were isobutane and water, mass spectral measurement was at m/e 220.

Arnold (93) described a G C /M S method for the determination of noscapine in opium preparations.

7.6. Chromatographic

7.6.1 Paper Chromatography

The paper-chromatographic method for the detection of alkaloids, e.g., codeine, veratrine, quinine and noscapine in several foods (a 100 g sample) is described. For the preliminary extraction of the alkaloids, add to the sample 300 ml of ethanol acidified to litmus paper with HCl. Digest on a water bath at 40° for 24 hr., filter and retain the filtrate. Add 100 ml of acidified ethanol to the residue, digest for another 12 hr. and filter again. Combine the filtrates and remove the alcohol by evaporating on a water bath at 40°. Add 30 ml of water and extract with diethyl ether (5 X 50 ml). Add NaOH soln. to the aq. layer till it is alkaline to litmus and extract with diethyl ether (5 X 50 ml). Evaporate the ether extract to 50 ml, transfer it to a separating-funnel

and wash with 2N HCl (3 X 20 ml). To the combined acid washings add NaOH till they are alkaline to litmus and extract with diethyl ether (3 X 50 ml). Evaporate the ether completely and dissolve the extract in 5 ml of ethanol. Retain this alcoholic soln. for chromatography by the descending technique on Whatman No. 1 paper. Apply drops of the test and standard soln. to the paper and impregnate its whole area above the starting line with a freshly prepared ethanolic soln. of formamide (1 : 1). Dry the paper between two filter-papers, then at 40° for 30 min. Spray the starting points with the ethanolic soln. of formamide (1 : 1) and after 10 min. transfer the paper to a glass container containing CHCl_3 at 25°. The development is complete in 2.5 hr. Dry the chromatogram at 105° and spray with a soln. of potassium iodoplatinate, washing off the excess with water in order to observe the spots on the white background. The following amounts of alkaloids can be detected -codeine 0.01 to 0.3 mg, quinine 0.005 to 0.015 mg, veratrine 0.1 to 0.15 mg and noscapine 0.05 to 0.075 mg. (56).

Table 5 describes methods used in noscapine analysis. Analysis of noscapine in opium by paper chromatography and spectrophotometry involved extraction of noscapine and measurement of the extinction of the spot at 290 m μ and comparison with standards (57). Other method was also described (58).

7.6.2 Thin Layer Chromatography

This technique has been used extensively for analysis of Opium and its preparations (63-71).

Table 6 - gives a resume of techniques and Table 7 - shows spray reagents and methods used.

Stahl and co-workers have proposed a standard procedure for the separation of opium alkaloids (63). Dried opium (0.1 g)

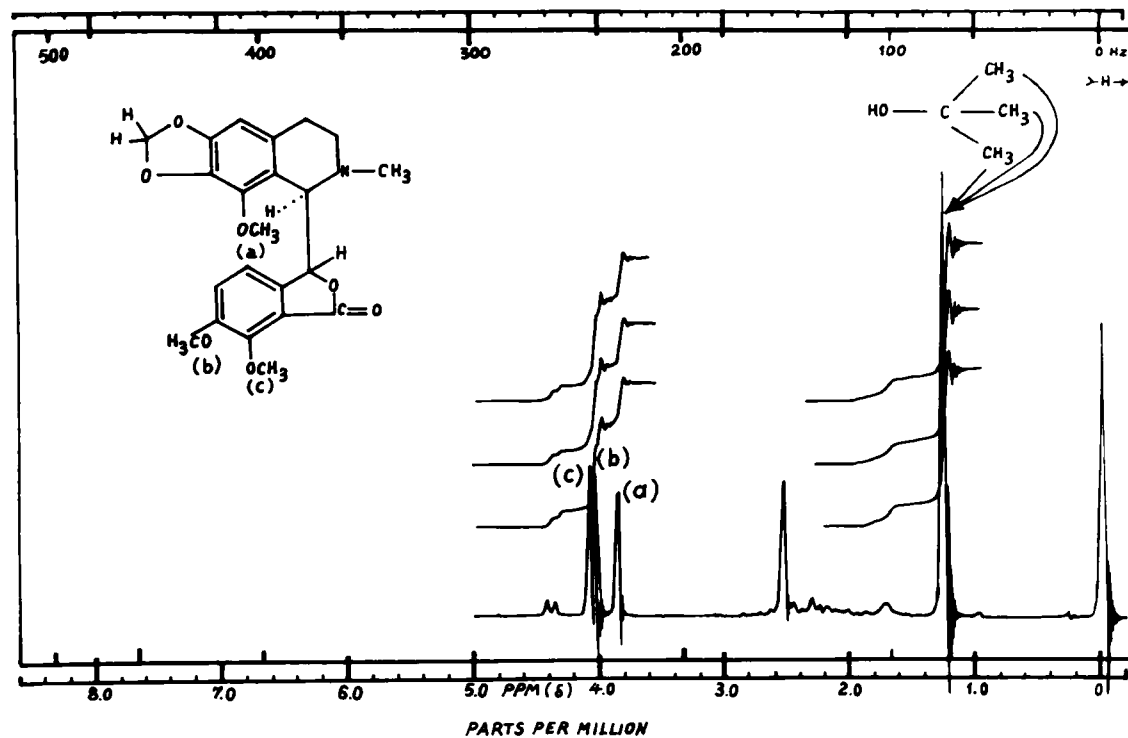


Fig. 11. PMR Spectrum of Noscapine, Tert-Butanol, and Tetramethylsilane in Ethanol-Free Chloroform.

Table 5. Paper Chromatography Used for Noscapine.

1	2	3	4	5	6
Stationary Phase	Technique	Mobile Phase	Rf.	Comment	reference
Paper	Two dimensional	1. Water sat. butanol-acetic acid 5:1 2. ether - 0.1 M acetic acid 5:2	- -	Alkaloids in Tetrapon "	(59)
Paper	Two dimensional	1. Dioxan-Formic acid-water 90:0.5 : 9.5 2. n-Butanol-acetic acid 5:1	- -	" "	(59)
Paper	One dimensional	1. Dioxan-Formic acid-water 90:0.5:9.5	-	"	(59)
Paper S & S 204 3b.	Ascending or descending	25% (NH ₄) ₂ SO ₄ in 0.5 N HCl	0.45	-	(60)
Whatman No.1 paper buffered at pH 3.5	Descending	Isobutylalcohol - toluene saturated with water 1 : 1.	-	-	(61)
Paper	One dimensional	Butyl acetate - butanol - acetic acid - water 47:9:28:16	-	Separation of noscapine from papaverine enhanced because of use of butyl acetate.	(62)

Table 5. (contd....)

1	2	3	4	5	6
Paper	One dimensional	Upper layer of a mixture n-butanol-acetic acid-water 5:1:4	-	-	(57)
Whatman No.1 impregnated above starting line with formamide- ethanol 1:1 then dried between filter papers then at 40°C for 30 min.	Descending	Chloroform		Starting points were sprayed with formamide in ethanol 1:1 after drying the paper	(56)

Table 6. TLC Techniques Used for Noscapine

Stationary Phase	Technique	Mobile Phase	Rf	Ref
Kieselgel HF ₂₅₄	Normal chamber	Toluene-acetone 95% ethanol 25% aq. NH ₃ 20 : 20 : 3 : 1	-	(63)
Silica gel G-Na ₂ CO ₃	"	Chloroform - Ethanol 4 : 1	-	(64)
Silica gel G.	"	Ethyl acetate	0.5	(72)
Kieselgel 60	"	Chloroform Benzene - Acetone 3 : 3 : 1	-	(73)
Silicagel G	"	Benzene-methanol 4 : 1	-	(65)
Silica gel G	"	Butanol-Acetic acid-H ₂ O 3 : 1 : 1	-	(66)
Silica gel G impreg. 4% Na ₂ CO ₃	"	Chloroform - Ethanol 4 : 1	-	(74)
Silica gel	"	Chloroform-isopropyl alcohol 10% aq. NH ₃ 30 : 10 : 1	-	(67)
Silica gel G	"	Benzene-methanol 4 : 1	-	(75)

Table 7. TLC Spray Reagents and Methods Used for Detection of Noscapine

Reagent	Procedure	Ref.
5% 3,5-dichloro-p-benzoquinonechlorimine in isopropyl alcohol	After spraying spray with aqueous NH_3 1 : 1 and observe in daylight and ultra violet.	(64)
4% $\text{Hg}(\text{NO}_3)_2$ in 3% HNO_3	After spraying the plate is heated 15 min. at 110°C and observed in daylight. Detection limit 2 μg	(65)
1. 3% H_2O_2 solution 2. 5% $\text{K}_4\text{Fe}(\text{CN})_6$ solution	Plate is first dried 10 min. at 100°C then sprayed with 1. then dried 10 min. at 100°C and sprayed with 2 and dried 10 min. at 100°C . Brown spots intensified to red. Detection limit 10 μg .	(66)
2.6 g $\text{CO}(\text{NO}_3)_2$ dissolved in 2 ml anhydrous acetic acid is added to 4.4 g of NaNO_2 dissolved in 10 ml H_2O then 20 ml acetic acid and 50 ml H_2O is added to mixture.	After spraying, the plate is heated at 105°C for 10 min. Spots are stable for several hours. Noscapine appears as blue-green fluorescent spot when viewed under ultra-violet light.	(74)
4% citric acid in acetic anhydride	Plate is heated at 80°C for 10 min. and viewed in daylight (detection limit 5 μg) and in ultra-violet radiation (detection limit 0.5 μg).	(75)

was powdered and triturated with 5 ml of 70% ethanol. The mixture was warmed at 50 - 60° for 30 min. then filtered and diluted to 10 ml with 70% ethanol. Tincture of opium (1 ml) was diluted with 9 ml 35% ethanol. Three portions (5, 10 and 20 μ l) of opium solution and similar portions of standard solution were applied to a layer of Kieselgel H F254 and chromatograph was developed to 15 cm with toluene - acetone - 95% ethanol 25% aq. NH_3 (20 : 20 : 3 : 1). For detection, the plate was heated at 110°C for 10 min. and the separated zones located in ultraviolet radiation. The spots are then sprayed using a modified Dragendorff's reagent and then with 0.05 N to 0.1 N sulphuric acid.

Also polyamide has been used as layer for chromatography (76). Where poly- ϵ -caprolactam resin (Amilan CM 10075) was used. Development for 2 hr. in cyclohexane-ethyl acetate - propyl alcohol - Me_2NH 30 : 2.5 : 0.9 : 0.1 showed noscapine at R_f 0.61 and development in H_2O -ethanol - Me_2NH 88 : 12 : 0.1 noscapine had R_f 0.00.

7.6.3 Gas Liquid Chromatography

Derivitization of samples included acetylation using acetic anhydride in pyridine (77) and treating sample with trimethylsilyl acetamide and trimethylchloroethane (68). Internal standards used were histapyrrodine hydrochloride, oestradiol valerate (78) Phenazone (79) and Squaline (77). Another method was also reported (80). Column types etc. are reported in Table 8.

7.6.4 High Performance Liquid Chromatography

Separations of pharmaceuticals combined in various formulations by HPLC on Sepherosil 5 μ m isocratically has been reported (81).

Noscapine in anti-cold preparations was separated on a column of Hitachi gel 3011-0 (82)

Table 8. GLC Conditions Used for Noscapine

Column Type	Stationary Phase	Liquid Phase	Carrier gas	Flow-rate	Temperature	Detector	Ref.
4 ft x 4 mm glass	Diaport S(80-100) mesh	3.5% SE-30	N ₂	30 cm ³ min ⁻¹	240°C	Flame ionization	(78)
1.5 m x 2.3 mm glass	Chromosorb G-HP AW-DMCS (80-100) mesh	0.75% HI-EFF 8 BP	N ₂	40 cm ³ min ⁻¹	150°C to 235°C at 1.25°C min ⁻¹	"	(79)
-	Supelcoport (80-100 mesh)	3% OV1	-	-	225°C-270°C at 2°C min ⁻¹	"	(68)
4 ft x 3mm od glass	Gas-Chrom Q (100-120 mesh)	2% OV 101	N ₂	30 cm ³ min ⁻¹	180°C 5 min. then 7°C min ⁻¹ to 250°C	"	(77)

using methanol : 28% aqueous NH_3 99:1 as eluting solvent and determination by spectrophotometry at 230 nm or 250 nm. Paracetamol, phenacetin, dipyrone, aspirin, caffeine, etenzamide and methylephedrine did not interfere. Other methods were also discribed (83, 84).

7.6.5 Ion-Exchange Chromatography

Knox and Jurand reported a method (85) for the separation of noscapine on dry packed column of Zipax SCX or SAX (37-44 μm) borate buffers at pH 9.2-9.8 containing 4% acetoni-
trile and 1% propanol were used at 500 to 1500 lb per sq. in. to elute noscapine.

7.6.6 Ligand-Exchange Chromatography

Poragel P.T. resin was used to separate alkaloids and noscapine has been analysed on this resin. 0.06 M-aqueous NH_3 in 33% ethanol was used as eluant and the elution volume for noscapine was 6.8 times the bulk column volume (86).

7.6.7 Partition Chromatography

In the assay of Tetrapon by partition chromatography the stationary phase is a phosphate buffer (87). 5 ml of 0.2 N NaOH is added to 0.4 g of papaveretum in 20 ml H_2O . The mixture is extracted twice with a mixture of 10 ml of chloroform in 30 ml ether and then with 10 ml of chloroform. The filtered extracts are evaporated to 0.5 - 1.0 ml and diluted with 25 ml of ether before transfer to the prepared column, 200 ml water saturated ether is used to elute noscapine. Other assay was also reported (88).

7.6.8 Paper Electrophoresis

Due to formation of molecular complexes between noscapine and 7-(2-hydroxyethyl) theophylline, tetramethyluric acid and 7-carboxymethyltheophylline, noscapine has

been separated from other isoquinoline alkaloids by reversed - phase paper chromatography and electrophoresis. Britton - Robinson buffer pH 3.5 to 4 was used as the mobile phase and o-xylene as stationary phase (89). Another method was also reported (1).

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PENICILLIN-G BENZATHINE

Franz Kreuzig

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1. DESCRIPTION

1.1. Name, Formula, Molecular Weight

Chemical Names

4-Thia-1-azabicyclo [3.2.0.] heptane-2-carboxylic acid, 3,3-dimethyl-7-oxo-6 [(phenylacetyl)amino]-[2 S-(2 α , 5 α ,6 β)]-, compd. with N,N'-bis(phenylmethyl)-1,2-ethanediamine (2 : 1), tetrahydrate.

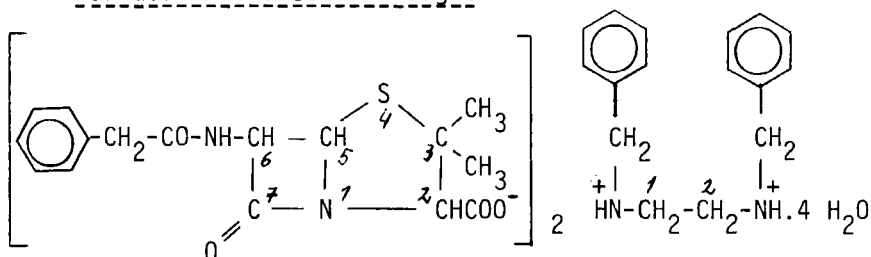
Generic names

Benzathine Penicillin G, Dibenzylethylenediamine-Dipenicillin G, Benzathine Benzylpenicillin, Penicillin G Benzathine.

Trade names

Tardocillin, Extencilline Panstriiline, Penidural, Citocillina, Diaminocillina, Wycillina, Beacillin, Penduran, Bicillin, Moldamin, Retarpen, Pronapen, Tri-Penilente, Debecillin, Retacillin, Penadur.

Formula and Molecular Weight



$C_{48}H_{56}N_6O_8S \cdot 4 H_2O$

Molecular Weight: 981,19

CA-Registry no. [1538-09-6]

1.2. Appearance, Color, Odor

White, odorless, crystalline powder.

1.3. Compendial References

Penicillin G benzathine is listed in the following compendia: The European Pharmacopoeia¹, the United States Pharmacopoeia with the Code of Federal Regulations² and the British Pharmacopoeia.³

1.4. Definition of International Unit

One mg of penicillin G benzathine represents 1211 penicillin units.

1.5. Content

The US Pharmacopoeia demands a content of not less than 57.9 % and not more than 71.6 % of penicillin G and not less than 1050 and not more than 1272 U/mg.

The European and British Pharmacopoeias refer to the substance without water. It should contain at least 96.0 % total penicillin and 24.0 to 27.0 % benzathine.

2. PRODUCTION

Szabo, Edwards and Bruce⁴ described a fractionated addition of a solution of benzathine acetate in water to a solution of penicillin-Na in water. When 60 % of the benzathine acetate is added, the precipitate is filtered and washed with water. To the filtrate are added 35 % of the benzathine acetate and the precipitate treated like before. Finally 5 % of the benzathine acetate are added to the filtrate. This fractionation method yields a microcrystalline powder. Dropping both moieties simultaneously into water, a product with crystals of about 100 μ m size will result after washing with acetone, water and drying.

When the precipitation medium contains formamide, the for-

mation of needles is prevented, which is important for parenteral application.⁵

For oral administration a product of inhomogeneous crystal form is obtained by recrystallisation from water and acetone.⁴

3. PHYSICAL PROPERTIES

3.1. Solubility

Penicillin G benzathine is practically insoluble in chloroform and ether. The solubility in water is rather low. Szabo, Edwards and Bruce⁴ determined the solubility in formamide, acetone, ethanol, benzene and water at different temperatures.

Solubility (mg/ml) of Penicillin G Benzathine in Formamide/Water Mixtures at Different Temperatures

Formamide [%]	273K(0°C)	298K(25°C)	315K(42°C)	333K(60°C)
100	21.2	28.0	92.5	158.0
95	3.2	3.8	12.0	43.0
90	3.2	3.2	10.3	31.6
75	1.4	1.4	3.6	13.2
50	1.0	1.0	2.5	5.1
25	0.3	0.4	0.7	2.0

Solubility (mg/ml) of Penicillin G Benzathine in Different Solvents at Different Temperatures

Temp. [°K(°C)]	acetone	ethanol	benzene	water
296 (23)	1.5	5.2	0.38	0.15
313 (40)	3.8	16.2	0.55	0.17
322 (49)	4.3	23.9	0.72	0.27
334 (61)	---	52.9	1.10	0.46

3.2. Melting Range

A decomposition point of 383 K (110°C) is given⁶, the determination of the melting point, according to L. Kofler, gives an interval from 385 - 388 K (112 - 115°C)⁷. Crystals, embedded in 1 drop of liquid paraffine, will decompose between 403 and 409 K (130 and 136°C).

Other authors⁵ found 383 - 408 K (110 - 135°C).

3.3. Optical Rotation

Penicillin G benzathine is dextrorotatory:

$$[\alpha]_D^{25} = + 206^{\circ} (c = 1, \text{methanol})^6.$$

3.4. Crystal Properties

These properties depend on the method of precipitation.

The crystals should have a shape so as to be suspended easily in a liquid carrier for injection. Adding formamide to the liquid for precipitation prevents the formation of needles and forms plate-like crystals, which can be broken to an average particle size of 150 μm .⁵

A method for the determination of the friability (in g/sec) was given.⁸ The importance of the angle of natural slope⁹, dispersion characteristics, the cohesiveness and wettability have been discussed¹⁰.

3.5. Ultraviolet Spectrum

A spectrum of a methanolic solution of penicillin G benzathine (500 $\mu\text{g/ml}$), obtained with a Zeiss DM 4 spectrophotometer, is shown in figure 1: the relevant parameter is the absorbance of the shoulder at 263 nm, which can be measured for spectrophotometric assay.

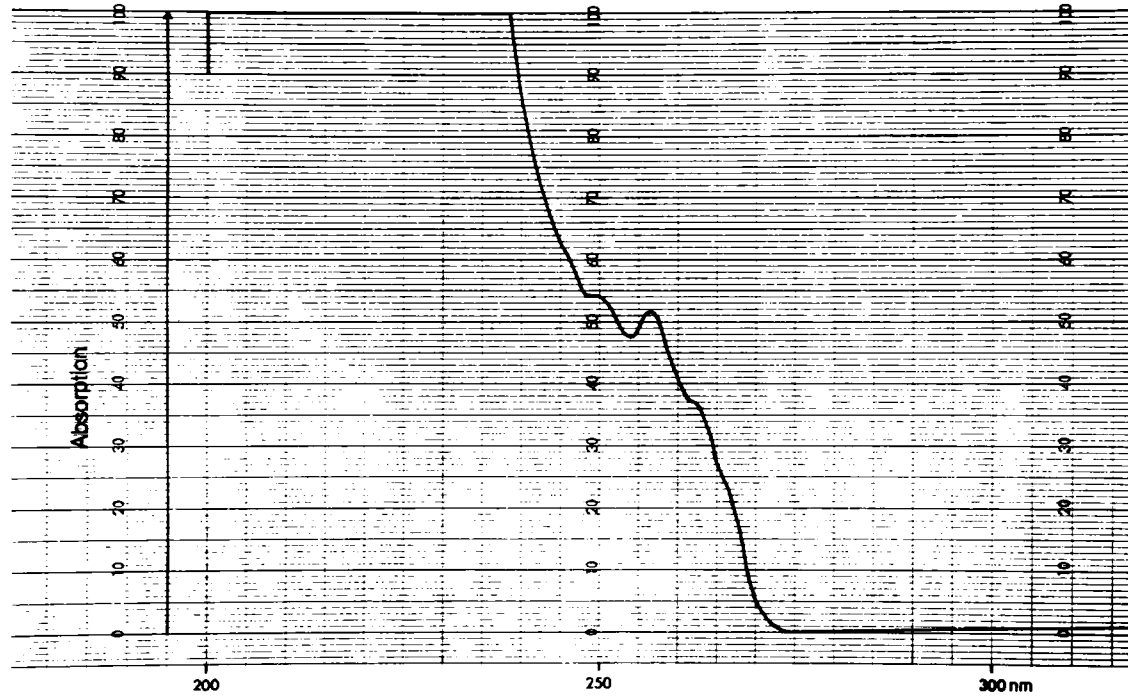


Figure 1: UV-Spectrum (see chapter 3.5.)

3.6. Infrared Spectrum

The infrared spectrum of penicillin G benzathine, obtained as a KBr-tablet (1.2 mg in 300 mg), was run on a Perkin Elmer 177. It is shown in figure 2.

The most significant stretch is that at 1780 cm^{-1} which is assigned to the β -lactam structure.

3.7. Nuclear Magnetic Resonance Spectrum

The proton NMR spectrum was obtained in DMF- d_7 solution containing TMS as internal reference, utilizing a Varian EM-390 NMR spectrometer operating at a frequency of 90 MHz (see figure 3).

Chemical shifts (δ , ppm)	Multiplicity	Intensity	Assignment
8.60	d ($J = 7\text{ Hz}$)	1 H	-CO-NH-
7.30	m	10 H	Phenyl-H
5.60	s	6 H	$^+\text{NH}_2 + \text{H}_2\text{O}$
5.50	2 d	2 H	H_5, H_6
4.20	s	1 H	H_3
4.10	s	2 H	Phenyl- CH_2 -N
3.70	s	2 H	Phenyl- CH_2 -CO
3.20	s	2 H	N- CH_2 - CH_2 -N
1.60	s	3 H	2 β - CH_3
1.50	s	3 H	2 α - CH_3

d = doublet, s = singlet, m = multiplet, J = coupling constant

These data are in good agreement with the paper of Wilson et al.¹¹

3.8. Mass Spectrum

The mass spectrum of penicillin G benzathine was run on a Varian MAT 311 in the field desorption mode (heating

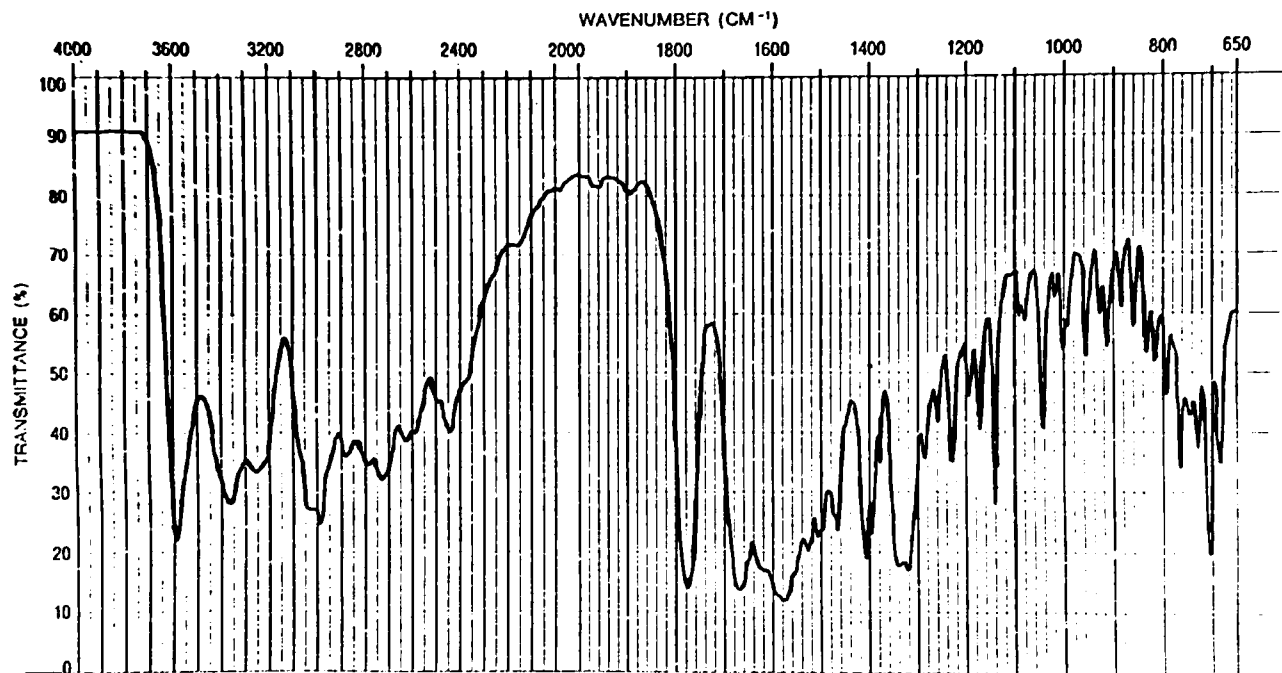


Figure 2: IR-Spectrum (see chapter 3.6.)

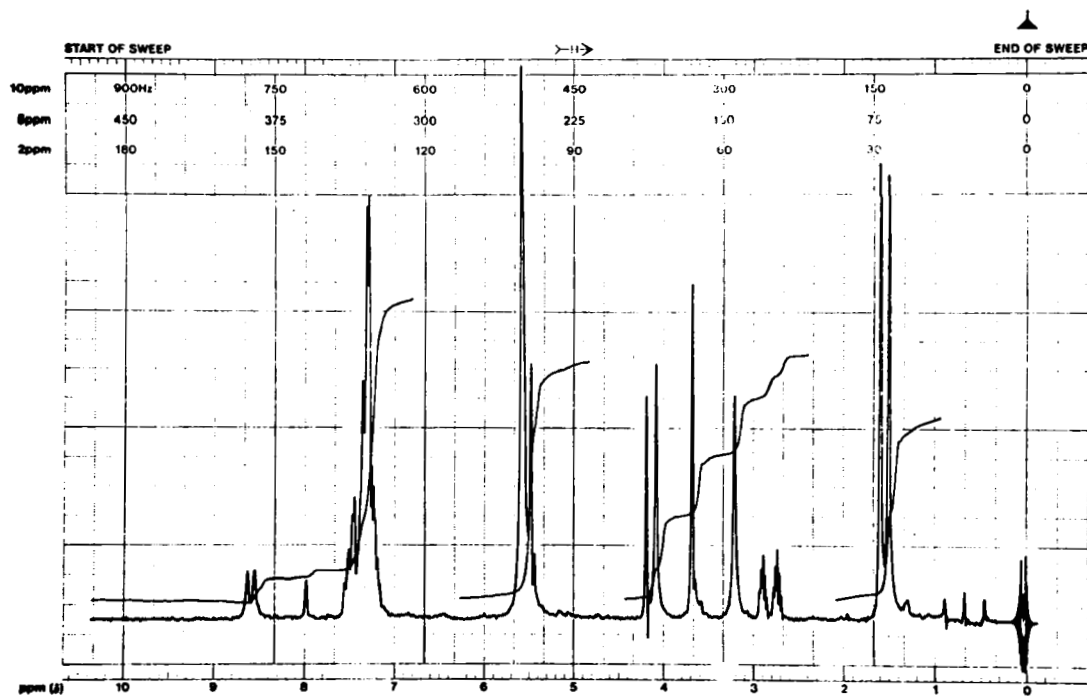


Figure 3: NMR-Spectrum (see chapter 3.7.)

current 10 - 20 mA). Intensive ions with the mass number of 575 can be attributed to penicillin G + benzathine + H^+ , whilst 910 corresponds to an associate composed of two penicillin G + benzathine + H^+ (figure 4).

4. STABILITY, DEGRADATION, ARTEFACTS

Penicillin G benzathine is very stable because of its low solubility in water and biological media, respectively. Being dissolved in aqueous media, the penicillin G moiety will degrade in a rather complex manner¹², which could be elucidated by NMR studies¹³.

These degradation patterns may be complicated by the aminolysis of penicillin G by the benzathine cation. It could be demonstrated that the reaction of 1,2-diaminoethane (monocation) with penicillin G gives an increased reaction rate compared with the monoamine of similar basicity. This is attributed to intramolecular general acid catalysis which in turn indicated that nucleophilic attack takes place from the least hindered α -side in disagreement with the prediction of the theory of stereoelectronic control¹⁴.

5. BIOPHARMACEUTICS

5.1. Pharmacokinetics

Because of its low solubility in water penicillin G benzathine is the longest acting penicillin; the resulting concentration in serum and urine respectively are, of course, rather low. On the other hand this drug offers, according to the amount administered, therapeutic penicillin G levels for about one month.

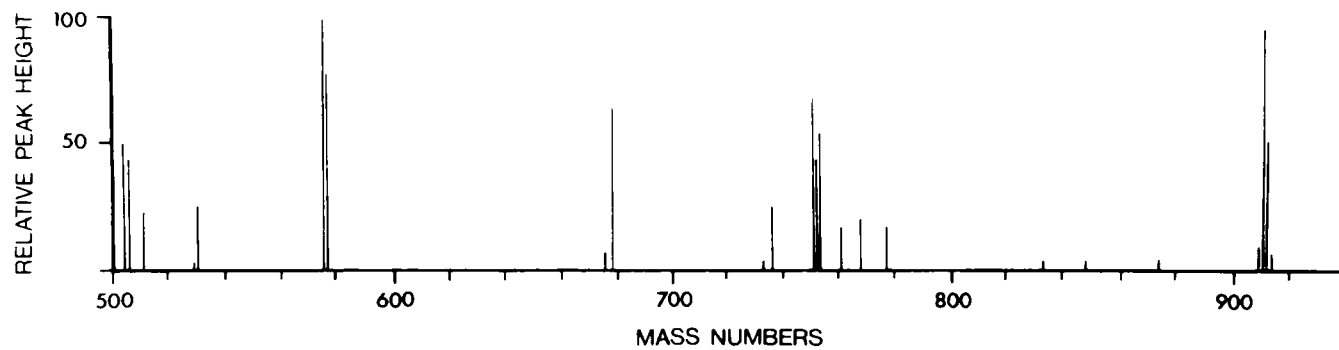


Figure 4: Mass-Spectrum (see chapter 3.8.)

Penicillin G benzathine, administered intramuscularly, is suited for the prophylaxis and long term therapy of syphilis and rheumatic diseases. Elias et al.¹⁵ followed serum penicillin levels in man after injection of this compound, and concluded that it showed no significant toxicity and gave serum penicillin levels of greater duration than any reported with other forms of repository penicillins.

When 2.400.000 IU of penicillin G benzathine are injected, the bioassay showed the existence of mean levels above 0.036 IU/ml serum and above 20 IU/ml urine for the duration of 26 days. The corresponding values for one hour after the injection were 0.3 and 234 IU/ml respectively.¹⁶

The injection of penicillin G benzathine suspensions may cause pain, therefore local anesthetics are added.

5.2. Metabolism

The penicillin G moiety of the drug decomposes in principal as mentioned in chapter 4.

6. METHODS OF ANALYSIS

6.1. Identification

The sample^{1,3} is shaken with 0.1 N NaOH, extracted with ether, evaporated, the residue is dissolved in ethanol. After the addition of picric acid, heating and cooling, the precipitated crystals are separated; their melting point is 487 K (214°C).

Simoes¹⁷ dissolved the sample in 0.2 M NaOH and extracted the benzathine moiety with ether. After evaporating the residue, HCl (10 %) and NaNO₃ (10 %) are added, the mix-

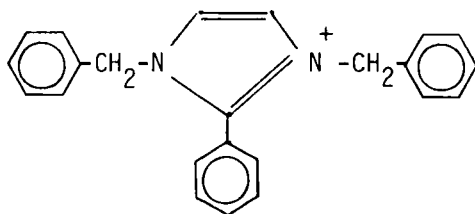
ture is extracted with ether, the ether is evaporated and the residue mixed with H_2SO_4 . A green color is observed. Addition of water gives a pink color, after adding NaOH the color changes to blue.

6.2. Iodometric Titration

The first method was described by Alicino¹⁸. Underivatized penicillin is inert to iodine in neutral aqueous solution. After hydrolysis with alkali, the resulting products consume 8 to 9 moles of iodine. The difference of the iodine consumption before and after hydrolysis is proportional to the quantity of penicillin. Penicillin G consumes 8.97 equivalents of iodine per mol; this ratio depends on the conditions of assay, therefore a blind sample has to be taken into account. The hydrolysis time is 15 - 30 minutes.

El-Sebai et al.¹⁹ described the analysis of penicillin G benzathine in 0.067 M Na_2HPO_4 solution with JCl in HCl-solution; the penicillin liberated was titrated with 0.05 M KJO_3 in $CHCl_3$; the end-point was the disappearance of the color from the $CHCl_3$ -layer.

The influence of iodine consuming impurities are discussed by LeBeille et al.²⁰ who proposed a modification of the iodometric method and found that benzathine and iodine form a benzimidazolium salt²¹



which interferes in the iodometric assay.

6.3. Nonaqueous Titration

For the determination of the benzathine moiety³ the substance is dissolved in a solution of NaCl and NaOH, extracted with ether, the combined extract is washed with water, the combined washings are extracted with ether, the extracts are evaporated to dryness, the residue is dissolved in anhydrous glacial acetic acid and titrated with 0.1 M HClO_4 , using 1-naphthol-benzein as indicator; the operation is repeated without the substance to be tested (blank value).

1 ml of 0.1 M HClO_4 is equivalent to 0.01202 g of benzathine.

6.4. Spectrophotometric Assay

Penicillin G in penicillin G benzathine is determined by measuring the extinction of a solution of 50 mg of sample in 100 ml absolute methanol at 263 nm in comparison to a reference standard².

Holbrook²² determined penicillin G benzathine after heating the substance with a Na-acetate/ CH_3COOH solution (pH = 4.6), containing a trace of CuSO_4 . The absorbance of the resulting penicillin acid is measured. The method has a standard error of 3 % and is applicable to preparations with a potency of at least 0.5 U/mg.

It has been found that benzathine forms a water soluble complex with Biebrich scarlet²³. The absorbance is measured at 516 nm and is related to the penicillin G benzathine content. The same authors²⁴ stated that penicillin forms a complex with methylene blue, which is measured at 655 nm and shows correlation with the penicillin G benzathine content.

For the determination of penicillin G benzathine in ointments the reaction with nitroprusside-Na and KMnO_4^{24} in alkaline solution produces a blue color. The sensitivity is 2000 U/ml of ointment.

6.5. Microbiological Assay

The sample is dissolved in methanol and further diluted with 1 % phosphate buffer pH = 6.4 so as to give a concentration of 1.0 U of penicillin G/ml. The agar diffusion assay is performed with *Staphylococcus aureus* (ATCC 29727)².

6.6. Thin-Layer Chromatography

Layer	Solvent Systems	Visualization	Ref
1. cellulose	CH ₃ OH/2-butanol/CHCl ₃ /HCl 10 % = = 10/10/10/1	J ₂	25
2. silica gel + cellulose	CH ₃ OH/1-butanol/CHCl ₃ /CH ₃ COOH = = 10/10/10/1	J ₂	26
3. silica gel	butyl acetate/CH ₃ COOH/MeOH/1-butanol/ phosphate buffer pH=7.3 = 80/4/5/15/20	FeCl ₃ + K ₃ [Fe(CN) ₆] + HCl	27
4. silica gel	acetone/HCOOH = 95/5	FeCl ₃ + K ₃ [Fe(CN) ₆] + H ₂ SO ₄	28
5. silica gel	isoamyl acetate/CH ₃ OH/HCOOH/H ₂ O (upper phase) = 65/20/10/5	NaNO ₂ + NH ₄ sulfamate + N-(1-naphthyl)ethylenediamine	28
6. silica gel	CH ₃ COOH/ butyl acetate/ 0.1 M phosphate buffer pH=5.6/1-butanol/CH ₃ OH = = 20/40/12/5/7,5	KJ + H ₂ PtCl ₆ + HCl - acetone	29

Comments:

- ad 1: The detection limits were in the range of 5-30 μg , it was stated, that cellulose has advantages over silica gel.
- ad 2: The authors claim the ease of the production of this plate and the specificity of the color of the spots after spraying.
- ad 3: With this method impurities and decomposition products were identified and quantitated visually.
- ad 4 and 5: Although this method is used for penicillin G procain, it refers to the decomposition of penicillin G in aqueous solutions to benzylpenicillic acid and benzylpenicilloic acid. Further decomposition of penicillin G occurs when alcoholic solutions are used for spotting the chromatograms, the corresponding alkyl- α -D-penicilloate being formed. The usefulness of different spray reagents is discussed.
- ad 6: This method allows for the detection of penicillic acid and penicilloic acid amongst the main components of penicillin G benzathine.

6.7. High Performance Liquid Chromatography

The paper of LeBeille et al.³⁰, concerning the HPLC determination of penicillin V benzathine, enables the separation of penicillin G from benzathine too, so it can be used for the indirect determination of penicillin G benzathine. The compounds are separated on a RP 8-column with the eluent $\text{CH}_3\text{OH}/0.05 \text{ M phosphate buffer pH}=3.5 = 53/47$, the UV-detector was set to 274 nm. Nachtmann and Gstrein³¹ have modified this method in the following

aspects:

Eluent: CH_3OH /phosphate buffer 0.06 M + 1 % triethylamine
→ pH = 5.5 = 30/70, T = 323 K (50°C), $\lambda = 220$ nm.

With increasing pH of the eluent the retention time of benzathine increases. This method offers the possibility of the quantitative determination of both moieties of the molecule.

Puttemans et al.³² have shown that the elution order of benzathine and penicillin can be changed by means of ion-pair HPLC, applying the eluent CH_3OH /phosphate buffer pH=3.0 = 30/70, adding $5 \cdot 10^{-4}$ M tetrabutylammonium-bromide.

7. ACKNOWLEDGMENTS

I am indebted to Dr. B. Prager, Biochemie GmbH, Kundl, for performing and interpreting the NMR-spectrum, to Dr. G. Schulz, Sandoz Forschungsinstitut GmbH, Wien, and Dr. A. Nikiforov, Organisch-Chemisches Institut der Universität Wien, for performing and interpreting the mass-spectrum.

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PHENYLBUTAZONE

Syed Laik Ali

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1. History

After second world war a German Chemist Hans Stenzl synthesised phenylbutazone, 1,2-diphenyl-4-n-butylpyrazolidin-3,5-dione in the research laboratories of J.R. Geigy in Basel, Switzerland (1). This compound formed water-soluble salts and was used in combination with aminophenazone in injection solutions. Phenylbutazone was brought in the market in 1952 as a single preparation under the trade-name of Butazolidin. It has remarkable antirheumatic and antiphlogistic properties.

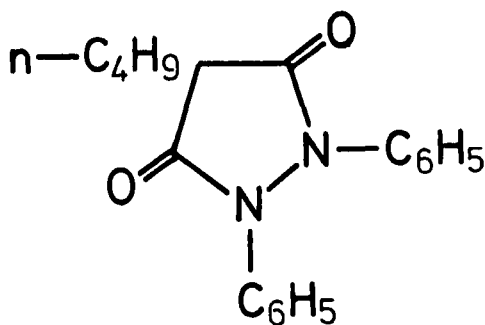
2. Description

2.1. Name, Formula, Molecular Weight

4-Butyl-1,2-diphenyl-3,5-pyrazolidinedione

$C_{19}H_{20}N_2O_2$

308.38

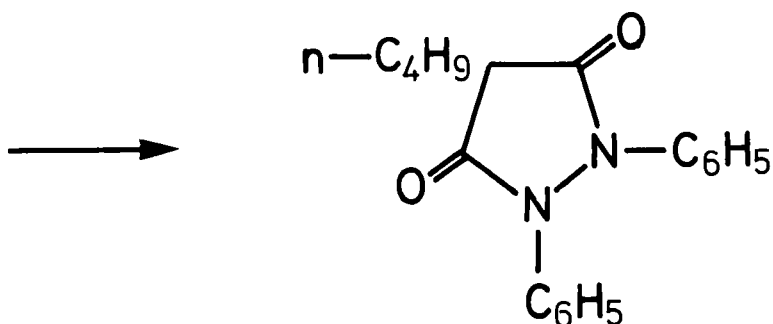
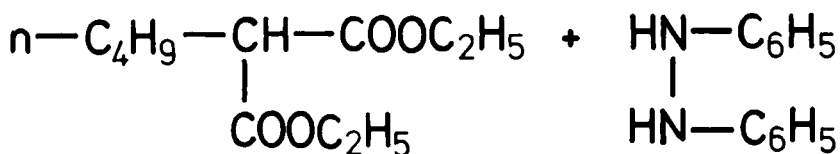


2.2. Appearance, Colour, Odour

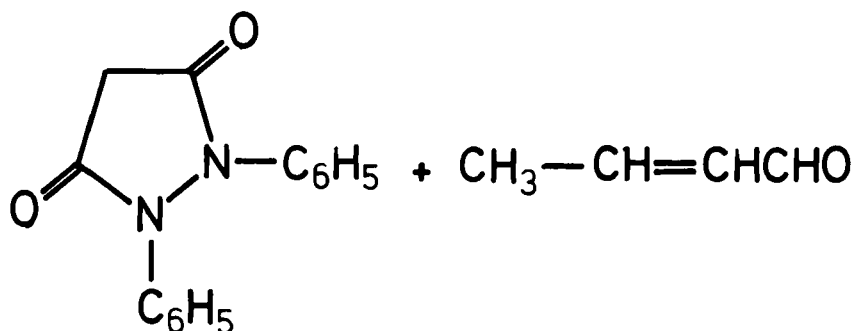
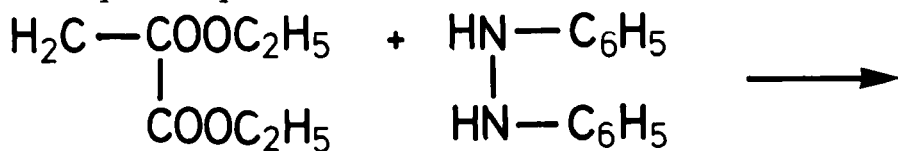
A white or almost white, crystalline powder, practically odourless, with a slightly bitter taste.

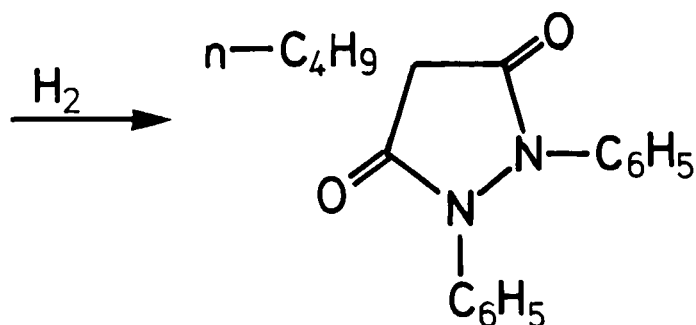
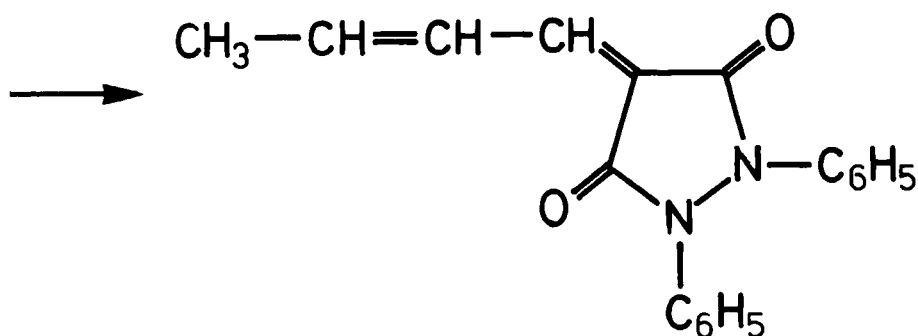
3. Synthesis

The first synthesis was carried out by heating n-butyl diethyl malonate with hydrazobenzene and sodium alcoholate (3).



Another method of synthesising phenylbutazone (3) consists of reacting diethyl malonate with hydrazobenzene which is further treated with crotonaldehyde and subsequently hydrogenated catalytically.





4. Physical Properties

4.1. Melting Range (4): Phenylbutazone melts between 104 and 107°C.

4.2. Solubility (5): The solubility of phenylbutazone in various solvents at 20°C is given in Table 1 as 1 part per specified parts-solvent.

TABLE 1
Solubility of Phenylbutazone at 20°C (5)

<u>Solvent</u>	<u>Solubility</u>
Water	practically insoluble
Ethanol	28 parts
Methanol	18 parts
Ether	15 parts
Chloroform	1.3 parts
Aceton	2.5 parts
Benzene	3.5 parts

4.3. Dissociation Constant

Phenylbutazone has an acidic hydrogen atom at C - 4. The Pk_a determined is reported to be 4.89 (6). Phenylbutazone is considered a carbon acid (carbon acids are acids in which the dissociating proton is bound to a carbon atom instead of heteroatoms such as oxygen or nitrogen) and the Pk_a value between 4.5 - 4.7 has also been given (7,11). The dependence of Pk_a values of phenylbutazone on the solvent medium has also been reported (8). Pk_a values in pure methanol, ethanol and water are given as 5.42, 5.76 and 5.07 respectively (8).

4.4. Loss on Drying

When dried in vacuum at 80°C and at a pressure of 30 + 10 mm of mercury for 4 hours it loses not more than 0.5 % of its weight (9).

4.5. Ultraviolet Spectrum (10)

Phenylbutazone in solution absorbs ultraviolet radiation to produce a spectrum with different maxima in different solutions. In methanol, 0.1 N HCl and 0.1 N NaOH it has absorption maxima at 243, 235 and 263 nm respectively. The corresponding molecular extinction coefficients and $A_{1\%}^{1\text{cm}}$ are reported to be (10):

	Methanol	0.1 N HCl	0.1 N NaOH
Absorption Maximum	243 nm	235 nm	263 nm
$A_{1\%}^{1\text{cm}}$	482	440	669
ϵ	14860	13570	20630

The UV spectra are given in Fig. 1. The absorption maxima in acid and alkaline solutions at 232 nm and 264 nm have also been reported (37).

4.6. Infrared Spectrum (10,11,62)

The infrared spectrum of phenylbutazone is given in Fig. 2. The spectrum was obtained with a Perkin-Elmer 257 spectrophotometer from a KBr pellet. There is a good agreement with the spectra reported in literature (10,11,62). The structural

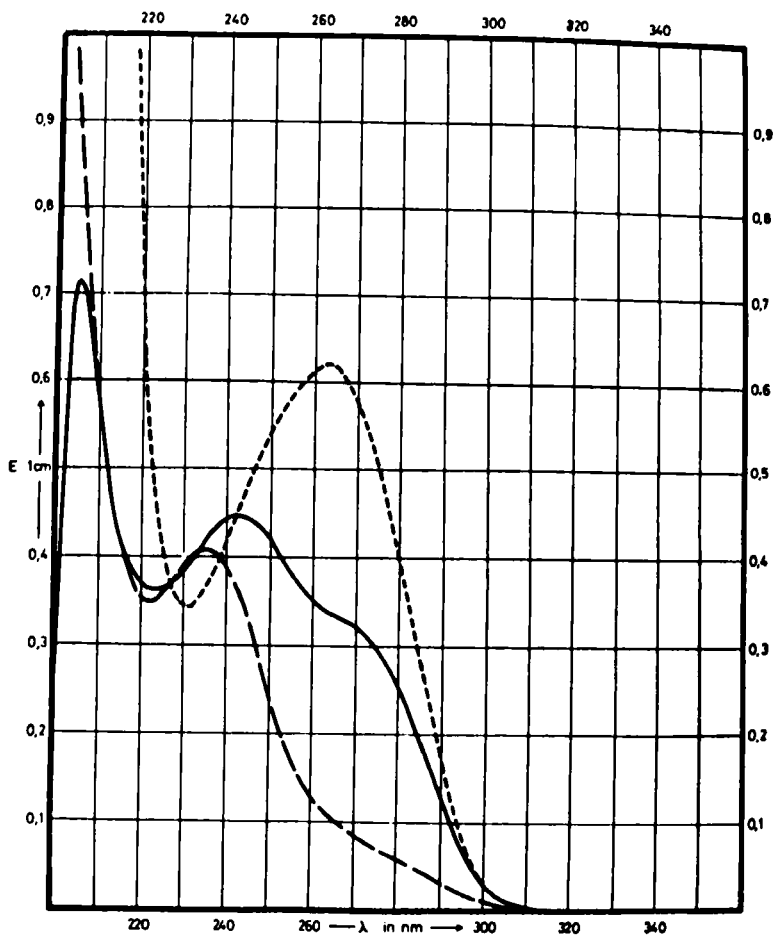


Fig. 1. UV Spectrum of Phenylbutazone in —(Methanol),
 --- (0.1N NaOH), - - (0.1N HCL)

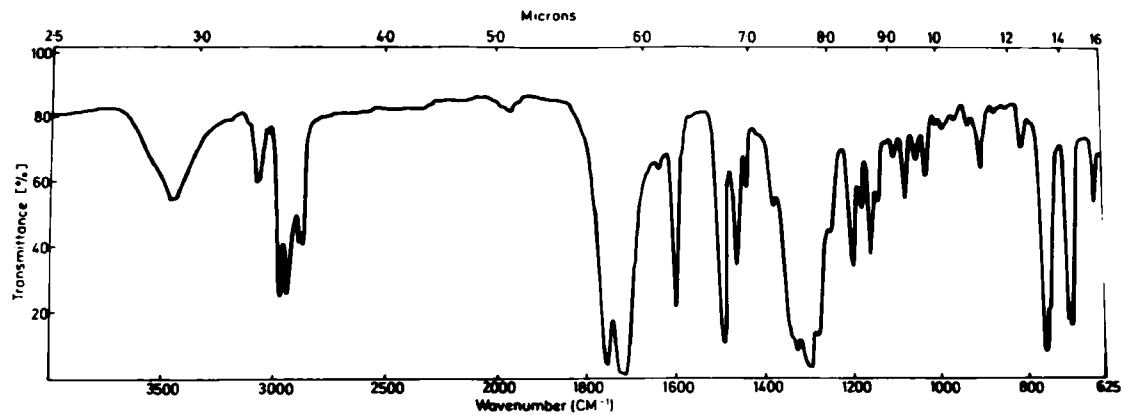


Fig. 2. IR Spectrum of Phenylbutazone, KBr Pellet, Perkin-Elmer 257 Spectrophotometer

assignments may be correlated with the following band frequencies.

<u>Frequency (cm-1)</u>	<u>Assignment</u>
1720 and 1755	Characteristic stretching vibrations of C = O group.
1600 and 1490	Characteristic skeletal stretching vibrations of the aromatic ring.
1300	Characteristic band of dioxopyrazolidine compounds.
760 - 695	Bands of monosubstituted phenyl.

4.7. Nuclear Magnetic Resonance Spectrum

The nuclear magnetic resonance spectrum of phenylbutazone , as shown in Fig. 3 was obtained on a Varian T-60 NMR spectrometer in deuterated chloroform containing 1 % tetramethylsilane as the internal standard. The following spectral assignments are made for Fig. 3:

<u>Chemical Shift (δ)</u>	<u>Assignment</u>
0.95 - 2 ppm doublet and multiplet	Protons of n-butyl rest.
3.35 ppm triplet	Proton at C - 4 position.
7.30 ppm multiplet	Aromatic phenyl protons.

There is a good agreement with the reported values in literature (62).

4.8. Mass Spectrum (12)

Mass spectrum is given in Fig. 4

Instrument: Varian CH5

Sample temperature (direct inlet): 80°C

Source temperature: 180°C

Electron energy: 70 eV

The prominent ions of this spectrum can be correlated to the structure as following:

m/e 308 = M	183 = C ₆ H ₅ NHNC ₆ H ₅
265 = M - C ₃ H ₇	105 = C ₆ H ₅ N ₂
252 = M - C ₄ H ₈	77 = C ₆ H ₅

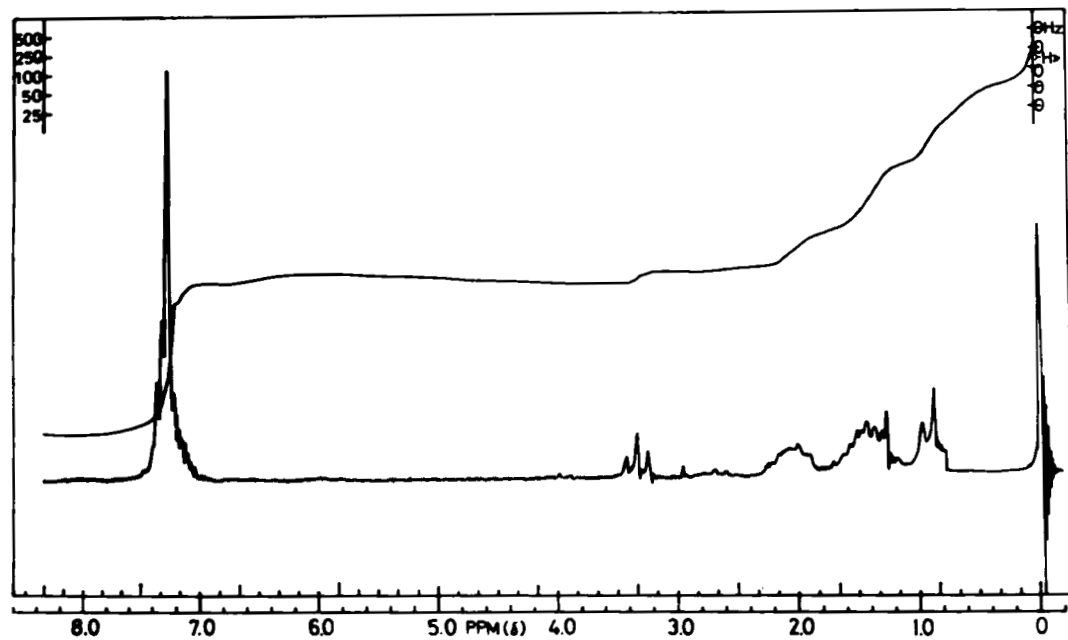


Fig. 3. NMR Spectrum of Phenylbutazone, Varian T60 Spectrometer.

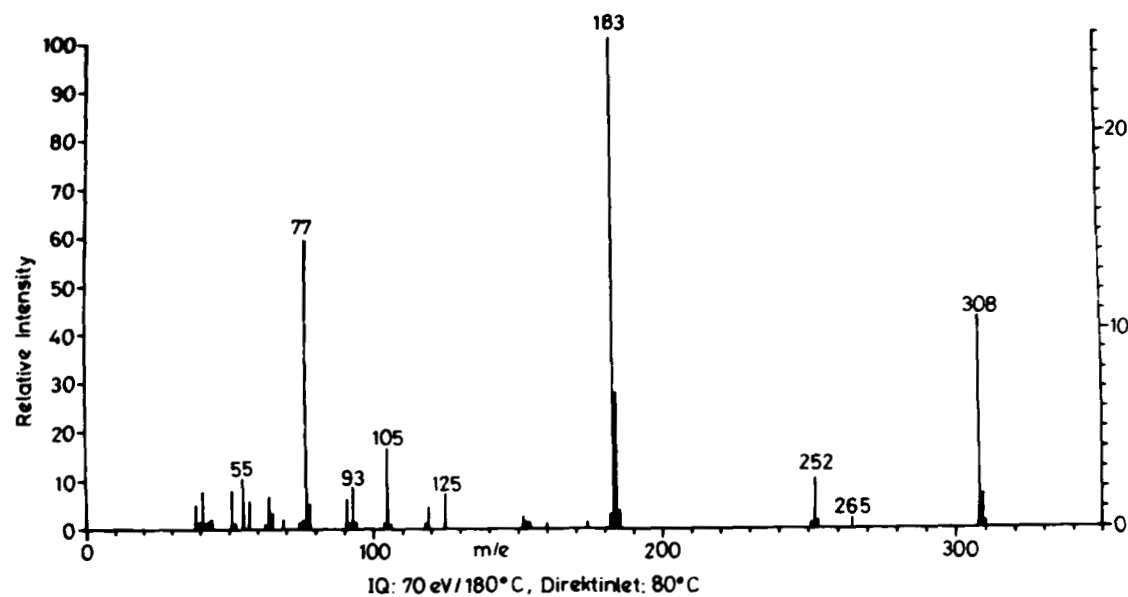


Fig. 4. Mass spectrum of Phenlybutazone

The elemental compositions of these ions have been determined by high resolution mass spectrometry (instrument: CEC 21 - 110) and are in agreement with the assignments made above (12).

The fragmentation pathways of Phenylbutazone and oxyphenbutazone were established by means of deuterium labeling, metastable peaks and accurate mass determinations. The major pathways are the McLafferty rearrangement of the molecular ion and formation of azobenzene and substituted azobenzene ions (13). The mass spectra of the methyl derivatives have also been discussed (13,14).

4.9. Differential Scanning Calorimetry (DSC), Differential Thermal Analysis (DTA), Thermogravimetry (TG)

The solid-solid transition of phenylbutazone polymorphs by heating was investigated by DSC and DTA (15). Three polymorphs of phenylbutazone were observed. Form I melted at 103°, Form II twice at 93° and 103° and Form III at 93° (15). Form II melted to an opaque paste at 93°, solidifying immediately and melting again at 103°C. Furthermore, the mutual transition phenomenon among these three polymorphs was observed by heating through DSC (15). In another investigation solid-solid and solid-liquid transitions of phenylbutazone polymorphs were investigated by DSC, DTA and TG (16). Four polymorphs were observed with transition points at 93.4, 95.1, 106.0 and 107.5°C (16). The curves are reproduced in Fig. 5. In addition two pseudopolymorphs from cyclohexane and isobutanol were determined containing one mole solvent to three moles phenylbutazone. The mutual transition behaviour of the polymorphs was investigated, the melting enthalpies determined and the energy of activation calculated from DSC-data. The DSC-curves of the different crystalline modifications were recorded on a Dupont 990 thermal analyzer, equipped with a DSC-cell 910 and calibrated with Indium (16). Thermal data of phenylbutazone polymorphs is given in Table 2, DSC and DTA/TG curves are reproduced in Fig. 6 (16).

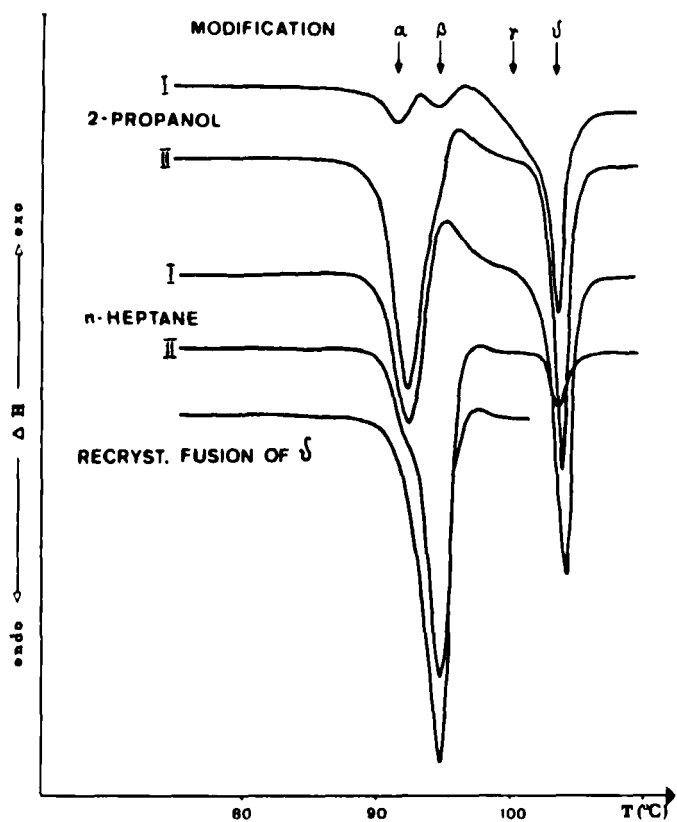


Fig. 5. DSC-curves of polymorphic forms of phenylbutazone and their mixtures obtained by crystallization from different solvents (heating rate $10^{\circ}\text{C}/\text{min}$, sensitivity 0.5 mcal/s in, sample weight 2-3 mg).

a) from cyclohexane; b) from isobutanol

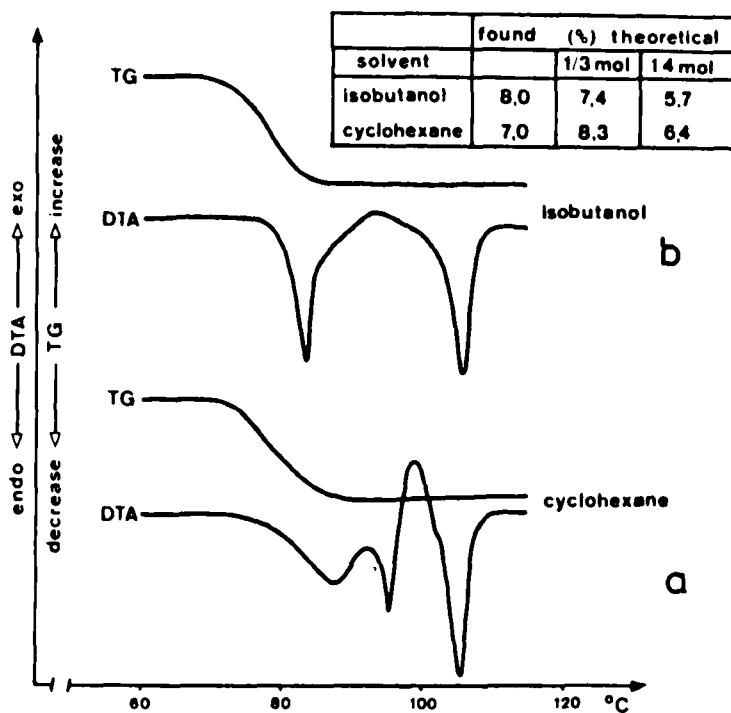


Fig. 6. Simultaneous DTA/TG-curves of phenylbutazone solvates

TABLE 2

Thermal Data of Phenylbutazone Polymorphs (16)

Polymorph	Transition temperature °C	Enthalpy of transition J/g
α	93.4	79.3
β	95.1	71.1
γ	106.0	-
δ	107.5	72.3

The thermal behaviour of the polymorphs under different treatment conditions has also been investigated (17). Compression of the thermodynamically unstable forms at a compression force of 1590 - 2040 Kg induced polymorphic changes in the crystals. Similar changes were also produced through grinding. The apparent equilibrium solubilities of 4 polymorphs at their transition temperatures were determined (17).

In Table 3 different transition temperatures and peak solubilities of the different polymorphs are given.

TABLE 3 (17)

Differential Scanning Transition Temperatures and Peak Solubilities of the Different Polymorphs (17)

Form	Solvent of Crystallization	Transition Temperature	Peak Solubility in Phosphate buffer, pH 6.95, at 36°C, mg/100 ml
I	Isobutyl alcohol	80°C	288.7
II	Cyclohexane	90°C	279.9
III	n-Heptane	93°C	233.6
IV	2-Propanol-Water	105°C	213.0

Polymorphism of phenylbutazone by a spray-drying method has also been investigated recently (18). DTA-curves of a spray-dried phenylbutazone from 5 % methylene chloride solution are given in Fig. 7 (18).

The investigation of a reference substance of phenylbutazone through DSC in a closed system gave following results (19):

Impurities	0.2 - 0.1 Mol%
Melting point	105.5°C
Latent heat of fusion	6.8 K cal./Mol

4.10. X-Ray Diffraction Spectrometry

The crystal structure of phenylbutazone has been determined with x-ray diffraction method (20,21). Thin needle-like crystals of phenylbutazone, obtained after controlled evaporation of an alcoholic solution were used for measurements (21). The unit cell dimensions of the crystals were determined from oscillations and Weissenberg photographs taken about crystallographic axes using nickel-filtered copper radiation. The cell dimensions of the crystals of phenylbutazone were refined on a computer-controlled Hilger and Watts 4-circle diffractometer. The crystal data for phenylbutazone is given in Table 4 (21).

TABLE 4
Crystal Data for Phenylbutazone(21)

Space group	P2 ₁ /c
a in Å	21.695 ± 0.004
b in Å	5.823 ± 0.002
c in Å	27.881 ± 0.004
β in degrees	108.06 ± 0.10
Volume of the unit cell in Å ³	3348.565
Molecular formula	C ₁₉ H ₁₉ O ₂ N ₂
Formula weight	307.168
No. of formula weights in the unit cell	8
Measured density in gm/cc	1.211 ± 0.020
Calculated density in gm/cc	1.218

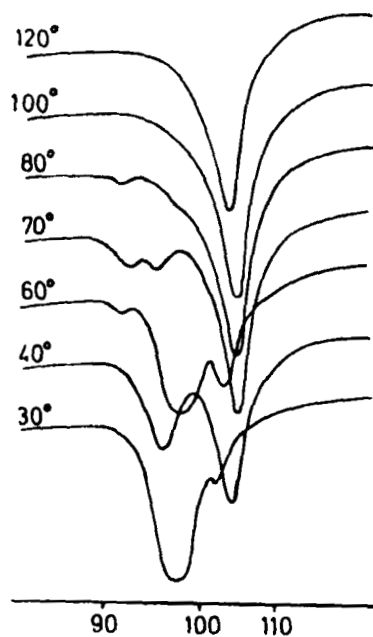


Fig. 7. DTA-curves of spray-cried phenylbutazone obtained at various drying temperatures. Numbers in the figure indicate drying temperature. Abscissa: temperature, °C.

Crystal structures of phenylbutazone and a 2:1 complex between phenylbutazone and piperazine are also reported (22).

X-ray powder diffraction patterns of phenylbutazone polymorphs and phenylbutazone solvates have been also investigated (16). The diffraction patterns of three modifications are given in Fig. 8 (16). X-ray diffraction patterns of the three peaks of phenylbutazone polymorphs are given in Table 5 (16).

TABLE 5
X-ray diffraction patterns of the three main peaks of the phenylbutazone polymorphs (16)

Polymorph	2θ	I/I ₀
α	8.7 ± 0.2	100
	19.4	70
	6.9	60
β	$8.35 \pm$	100
	20.2	70
	7.05	60
γ	7.9 ± 0.2	100
	20.7	70
	7.05	60

4.11. Scanning Electron Microscopy (16,17)

Photomicrographs were made using a Jeol JSM - U3 scanning electron microscope. Prior to investigations the substances were coated with gold using a direct coating sputter technique giving a coating layer of about 500 Å. A photograph of polymorph modification is given in Fig. 9 (16).

5. Colour Reactions

When phenylbutazone is heated with glacial acetic acid and hydrochloric acid, hydrazobenzene is formed immediately which rearranges itself to benzi-dine. The blue or violet colour yielded is due to oxidation of hydrazobenzene. After addition of sodium nitrite and pouring into a solution of sodium carbonate and β -naphthol a red product is formed, which mainly consists of 4,4'-bis (2-hydroxyl-1-azonaphthyl) biphenyl and 2-hydroxynaphthyl-1-azobenzene (23). Red colouration is produced when

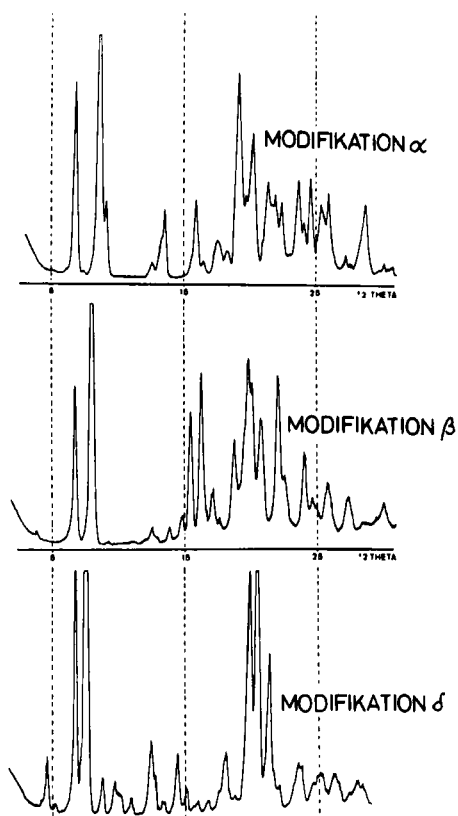


Fig. 8. Powder X-ray diffraction of phenylbutazone polymorphs.

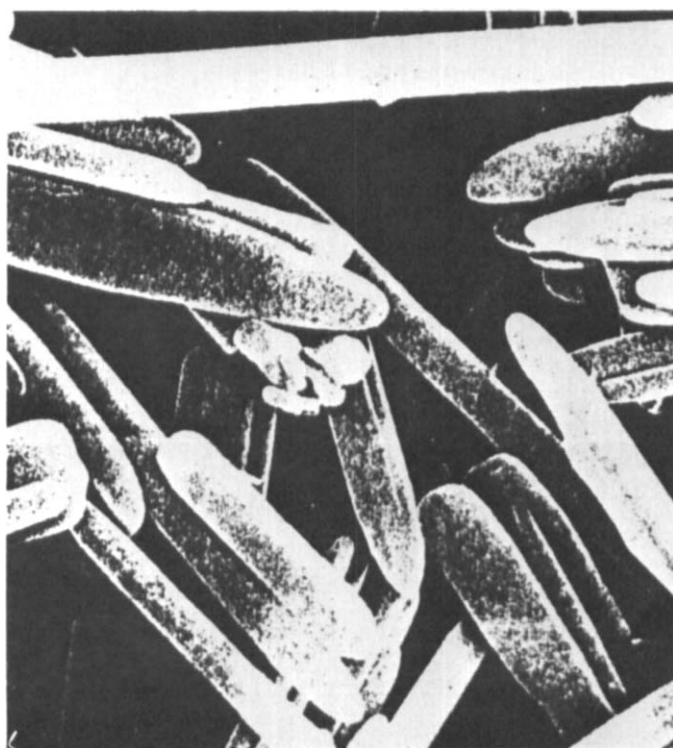


Fig. 9

Scanning Elektron Photomicrograph of
Phenylbutazon;

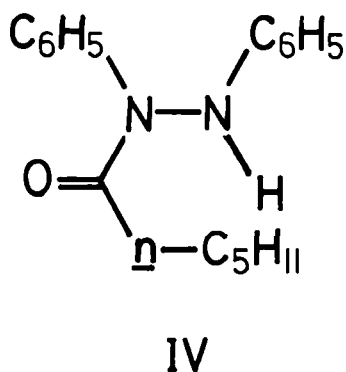
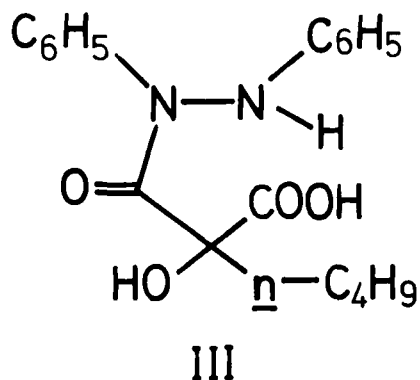
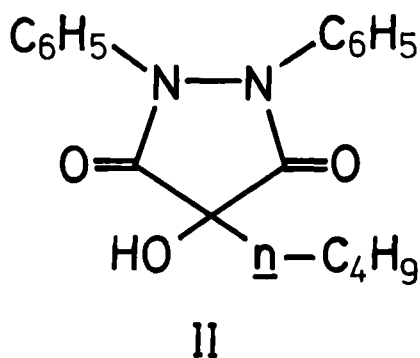
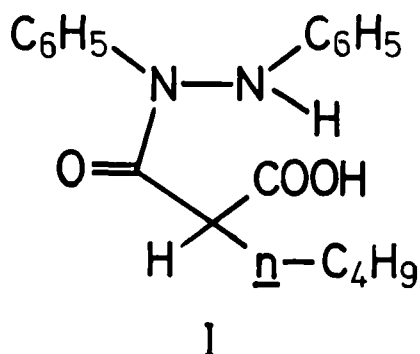
Polymorph δ magn. 1200 x

sulfuric acid and potassium nitrate are treated with phenylbutazone which turns to orange-red on reaction with ammonia solution (24). Vanadium-sulfuric acid solution reacts with phenylbutazone to give a dark green colouration (25). An alcoholic solution of iron (III) chloride and 2,2'-dipyridyl reacts with phenylbutazone to give a red colouration (26). Hydrolysis of drug with phosphoric acid followed by the diazotization and coupling of the resulting products with β -Naphthol gives a colour reaction (27).

6. Degradation and Stability

Aqueous solutions of phenylbutazone sodium decompose in course of hydrolysis and oxidation, for example with sodium hydroxide and hydrogen peroxide (28,29). Degradation occurs also in oxygen-free basic solutions and in oxygenated solutions (30,31). Degradation pathways in other solvents such as N, N-dimethylformamide, N, N-dimethylacetamide, diethyl carbonate and propylene glycol-water have also been described (32). Four mainly occurring decomposition products of phenylbutazone are the following (29):

1. N-(2-Carboxycaproyl)-hydrazobenzene (Carboxylic acid of phenylbutazone) (I)
2. 4-Hydroxyphenylbutazone (II)
3. N-(2-Carboxy-2-hydroxycaproyl)-hydrazobenzene
(α -hydroxycarboxylic acid of Phenylbutazone) (III)
4. n-Caproylhydrazobenzene (IV)



The oxidation product of phenylbutazone sodium in aqueous solution is 4-hydroxyphenylbutazone (II), the product of hydrolysis is N-(2-carboxycaproyl)-hydrazobenzene (I). Under the action of hydroxyl ions on 4-hydroxyphenylbutazone N-(2-carboxy-2-hydroxycaproyl)-hydrazobenzene (III) is formed which leads after further hydrolysis to n-carpropylhydrazobenzene (IV). This could be further oxidised giving a mixture of cis and trans-azobenzenes (29). Phenylbutazone forms in aqueous and partially aqueous solutions by a reversible reaction the compound I (33). The reaction rate and the position of equilibrium depend on the solvent, but practically not on the pH (33). The temperature dependence of rate constants is given in Table 6 (33).

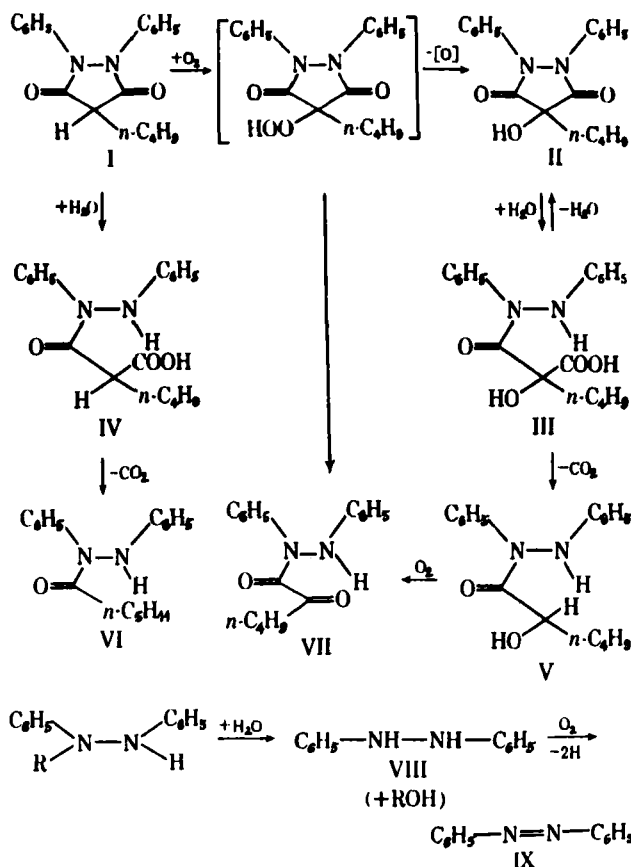
TABLE 6

Temperature Dependence of Rate constants; Phenylbutazone
concentration 0.6 M (33)

Medium	Temp.	$k_1 \times 10^6$	$k_2 \times 10^6$	K	$k_3 \times 10^6$
	$^{\circ}\text{C}$	(s^{-1})	(s^{-1})		(s^{-1})
H_2O	70.5	1.39	0.20	6.9	0.030
	80.1	3.55	0.58	6.1	0.124
	90.8	9.94	1.70	5.8	0.53
	100.0	22.8	4.39	5.2	1.98
56% Triethylene- glycol	60.4	0.087	0.076	1.1	-
	70.9	0.297	0.33	0.9	0.33
	78.9	0.750	0.81	0.9	0.95
	89.7	2.12	-	-	3.83
	100.6	5.84	-	-	12.4
35% N-Methyl-2- pyrrolidon + 15% Triethylene- glycol	60.2	0.061	0.080	0.77	-
	70.1	0.198	0.319	9.62	-
	80.7	0.620	1.20	0.51	-
	91.0	1.55	3.56	0.44	7.8
	100.8	4.06	10.9	0.37	23

The first three compounds are colourless and may be extracted with ether from acidified solution. The coloured product *n*-caproylhydrazobenzene may be isolated after extraction with chloroform (28). The preparation and separation of decomposition products of Phenylbutazone has been reported in detail by Pawelczyk and Schmid (28,29,33). The UV-spectra of Phenylbutazone and its decomposition products in 0.001% ethanol solution are given in Fig. 10 (28).

The degradation pattern of Phenylbutazone has been given by Awang as following (38):



The oxidative degradation of phenylbutazone with an acidic potassium permanganate solution and an alkaline hydrogen peroxide solution has also been reported (34). Mass spectral behaviour of decomposition products of phenylbutazone has also been investigated (35,13). Two previously reported but unidentified phenylbutazone degradation products were isolated by chromatography and identified by mass and nmr spectrometry (36).

Beckstead (37) has examined the decomposition of phenylbutazone in solid dosage forms. Awang (38) has tested 56 samples of phenylbutazone tablets and 15 samples of phenylbutazone-antacid formulations in the form of capsules and tablets. The degradation of phenylbutazone bulk drug was observed under conditions of accelerated hydrolytic and oxidative decomposition. In presence of magnesium carbonate phenylbutazone is unstable to heat due to chemisorption (39). The photodegradation of phenylbutazone in aqueous solution leads to 2-oxocaproic anilide and other compounds. In the methanolic solution n-butyl (methoxy) malonic dianilide is obtained. In aqueous solution in presence of diethylamine the reaction leads to n-butyl-diethylaminomalonic dianilide and other compounds (40).

Phenylbutazone formulations showed no evidence of chemical instability when stored at ambient temperature, 37°C and 37°C with 75% relative humidity. Measurable chemical degradation occurred only at 60°C, with several formulations showing more than 50 % degradation. The extent of degradation can vary among the tablets of the same bottle and between bottles of the same lot (41).

Chemical degradation occurs at 37°C also in some phenylbutazone-antacid formulations and was common at 50°C and 60°C (41). Phenylbutazone tablets are also subject to physical instability which is manifested in a decrease of dissolution rate probably due to polymorphism of phenylbutazone (17) or to change of the properties of excipients such as gelatine and or acacia subcoats of sugar-

coated tablets (42). The stability of phenylbutazone in solid dispersions containing polyethylene glycol 6000, urea and polyvinylpyrrolidone was investigated by subjecting the samples to accelerated storage conditions. The decomposition of phenylbutazone increased in presence of PEG 6000 and urea, whereas polyvinylpyrrolidone had no significant effect on the stability of the drug. The effect of humidity on the stability of phenylbutazone formulations was less pronounced than the temperature (43,44). The stability of phenylbutazone is also impaired at 50°C when formulated with polysorbate 80 (44). In suppository formulations degradation products of phenylbutazone were also identified and the process was described as mainly oxidative (45,46).

7. Dissolution

In respect of dissolution rate of phenylbutazone PEG 6000 and urea had an adverse effect but polyvinylpyrrolidone was considered to be a superior carrier (43). The initial dissolution rate of phenylbutazone and deuterated phenylbutazone (d-phenylbutazone) into a 25 % (v/v) ethanol-water solvent at 25°C and constant ionic strength from a constant-surface area pellet was studied as a function of apparent pH, buffer concentration and stirring rate (47). The same author (47) has postulated that phenylbutazone may have a hindered dissolution due to simultaneous reversible noninstantaneous chemical reaction which must take place in the aqueous diffusion layer if the dissolution involves rate-determining diffusion through the aqueous diffusion layer. Other authors (48) have investigated the behaviour of phenylbutazone in its transfer through a dimethylsiloxane membrane as a function of pH value.

The presence of surfactants in phenylbutazone formulations caused an increase in the initial dissolution rate. The dissolution decreased after Storage (44).

The dissolution rates of various polymorphic modifications of phenylbutazone has been subject of number of studies (15,16,17,18). Dissolution studies were conducted in a 0.2 m aqueous phosphate buffer of pH 7.5 with the disc method.

α and β forms show a slight transition into δ when they are compressed into discs. The dissolution rate of the β -modification is higher than all other modifications (16).

The dissolution rates of various phenylbutazone polymorphs in 0.2 m phosphate buffer, pH 7.5 at 37°C with disc method at 100 rpm are given in Fig 11 (16). In another study (18) the dissolution rates of spray-dried phenylbutazone samples at various drying temperatures were measured at 37°C in the USP dissolution test solutions. The solubility of the samples obtained at 30°C was 1.5 times higher than that of the sample obtained at 120°C and the bioavailability of the new form crystals would be expected to compare with the other known forms (18).

8. Methods of Analysis

Titrimetry

Titration of phenylbutazone dissolved in acetone and with an indicator bromthymol blue with sodium hydroxide has been adopted as the official method of analysis in European Pharmacopoeia (49). In USP XX (4) titration of phenylbutazone is performed with 0.1 N tetrabutyl ammonium hydroxide. Determination of end-point is done potentiometrically using a glass-calomel electrode system. These methods are not specific, as the common decomposition products such as the carboxylic and hydroxy carboxylic acids are also titratable. Bromometric assay with a potentiometric end-point determination has also been performed (50). Volumetric determinations of phenylbutazone with chloramin-T (51) and iodine-chlorine solutions are also known (52). The official BP method involves a non-aqueous titration with acetone as solvent (53). A non-aqueous titration in tetramethyl urea as solvent has also been reported (54). The use of

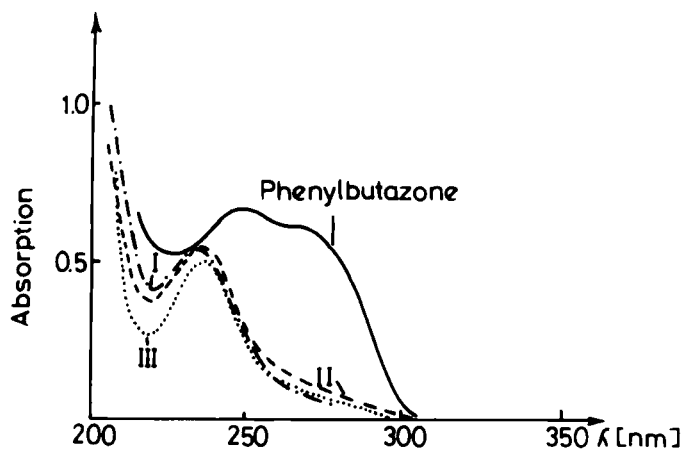


Fig. 10. UV spectra of phenylbutazone and its decomposition products in 0.001% ethanol solutions, phenylbutazone, I, II, III.

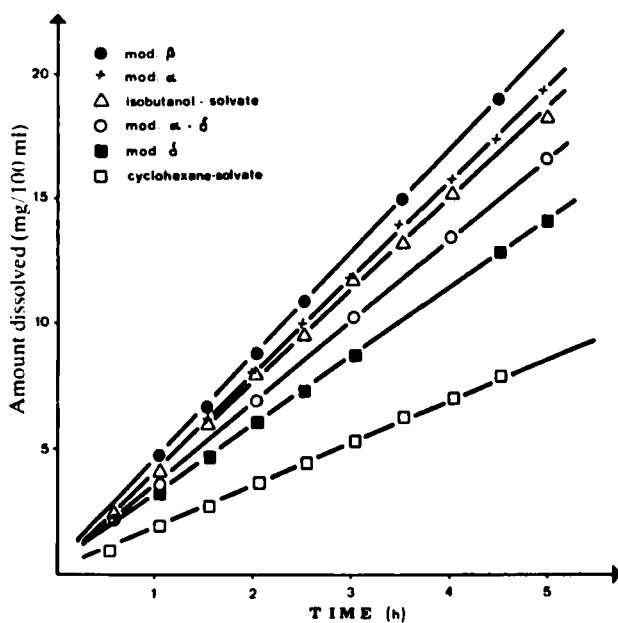


Fig. 11. Comparison of dissolution rates of phenylbutazone polymorphs in 0.2M phosphate buffer pH 7.5 at 37°C and 100 rpm.

borhydrides of alkaline metals for the volumetric analysis of phenylbutazone is also known (55).

Gravimetry

Phenylbutazone has been determined gravimetrically after precipitation with xanthidrol (56,57).

Colorimetric Analysis

Determination at 750 nm after reaction with phosphor-molybden-tungstic acid (58), at 520 nm after reaction of phenylbutazone with iron (III) chloride and 2,2'-dipyridyl have been reported (26). Hydrolytic conversion of phenylbutazone to benzidine and its subsequent diazotization and coupling reaction has also been utilised for its colorimetric determination (59).

UV-Spectrophotometry

Phenylbutazone can be determined spectrophotometrically after oxidation to azobenzene at 320 nm (60). A uv-spectrophotometric measurement of phenylbutazone in presence of decomposition products, other medicinal agents and interfering dyes involves an acid and base shake-out followed by measurement at 232 and 264 nm (37). Molar absorptivity values of phenylbutazone and its decomposition products in neutral and alkaline solutions are given in Table 7 (37).

Chromatographic Methods

Paper Chromatography

Paper of Schleicher and Schüll Company as stationary phase and butanol, formic acid and water (12+1+7) or butanol, ammonia 25% and water (84+8+8) as mobile phases have been suggested for the paper chromatographic separation of phenylbutazone (61). Detection was performed with Dragendorff's reagent, diazotized sulfanilic acid and 1% mercury (II) nitrate solutions (61).

Thin Layer Chromatography

Several systems for the TLC analysis of phenylbutazone and its decomposition products are available. Backstead (37) has reported cyclohexane-chloroform-methanol-acetic acid (60+30+5+5) as solvent system and silica gel GF thin layer plates as stationary phase to be ideal for resolving and semiquantitatively estimating

TABLE 7
Molar Absoptivity Values of Phenylbutazone and its
Decomposition Products (37)

Compound	Neutral Solution		0.01 N N NaOH		0.1 N NaOH
	λ_{max} nm	ϵ_{max}	λ_{max} nm	ϵ_{max}	$\epsilon_{264 \text{ nm}}$
Phenyl butazone	247	15360	264	20925	20925
Decomp. Prod. I	236	18810	232	15425	3550
Decomp. Prod. II	236	17385	232	16400	3835
Decomp. Prod. III	236	19000	232	16550	3915
Decomp. Prod. IV	236	18455	236	14890	3080

* Decomposition Products I, II, III and IV are the same as mentioned under 6 (Degradation and Stability)

phenylbutazone and its decomposition products. A solvent system of cyclohexane-methyl ethyl ketone-chloroform-acetic acid (39+62+3+10) and silica gel GF 254 thin layer plates were also found to be suitable (62). Several other solvent systems and silicagel HF and GF₂₅₄ thin layer plates have been recommended for the TLC of phenylbutazone (61,63,64,65). Silica gel TLC plates previously treated with a 2% solution of sodium hydrogensulfite deters the formation of 4-hydroxyphenylbutazone during the process of development of thin layer plates in solvent system (66). On-plate oxidation of phenylbutazone was effectively suppressed by utilising a 1 : 1 mixture of kieselgur and silica gel and impregnating the TLC support with McIlvaine buffer, pH 6.0 (37). Silica gel TLC folies and benzene - acetone (80+20) solvent have been used to separate phenylbutazone from ketophenylbutazone (67).

Detection of phenylbutazone and its decomposition products can be achieved by viewing the plates under uv-light. Spraying the TLC plates with chlorine-o-toluidine reagent gives blue to violet coloured spots with a sensitivity well below 1 microgram (37). Folin-ciocalteu reagent gives also coloured spots and is useful in indicating the presence of components which could be overlooked by both uv-light and chlorine-otoluidine reagent (37). Phenylbutazone can also be detected with iron (III) chloride in hydrochloric acid, Dragendorff's reagent and dimethylaminobenzaldehyde solution or subjecting the TLC plates to iodine or chlorine vapours (62). Another spray reagent used was 0.5% potassium dichromate in 20% sulfuric acid (38). A quantitative densitometric determination of decomposition products of phenylbutazone at 232 nm with a Zeiss KM 3 Chromatogramm-Spektrometer has also been reported (66).

Gas Chromatography

Anachrom ABS/QF-1 glass column at 200°C isotherm with nitrogen as carrier gas were used for analysing phenylbutazone through gas chromatography (62). Perego (68) has given a gas chromatographic method of determination of phenylbutazone in biological fluids in presence of its metabolites

1-phenyl-2-p-hydroxyphenyl-3,5-dioxo-4n-butylpyrazolidine and 1,2-diphenyl-3,5-dioxo-4-(3-hydroxybutyl)-pyrazolidine. A 2.5% SE 30 on Gas Chrom Q, 100-120 mesh glass column was used with nitrogen as carrier gas and flame ionization detector. Quantitation was done by using promazine as an internal standard. Phenylbutazone was extracted from the rat serum or urine after acidifying with 1 N HCl and extracting with n-heptane (68). Ali (69) has separated phenylbutazone and three of its decomposition products I, II and III on a 2 m steel column, packed with 3% SE 30 on Varaport 30, 100-120 mesh. Linear temperature programme was run between 190-250°C, 6°C/min. Injector and detector temperatures were 230°C and 270°C respectively and nitrogen was used as a carrier gas and flame ionization detector. Mostly decomposition product I was found in phenylbutazone formulations (69). A derivatization procedure for phenylbutazone and other acidic drugs after extraction from plasma and prior to its gas chromatographic determination has been given by Roseboom (70). Phenylbutazone has been chromatographed as its n-butyl ester after treating it with n-butyl iodide in presence of N, N-dimethylacetamide in a methanolic tetramethyl ammonium hydroxide solution. 1.5 m glass columns packed with 3% OV 1, 3% OV 17 and 3% SP 1000, all on chromosorb WHP, 100-120 mesh, were used for the determination of phenylbutazone at 230, 250, and 270°C column temperatures isotherm respectively. The injection port and detector (FID) temperatures were 30°C higher than the column temperature. The retention times of phenylbutazone on these three columns were found to be 220, 280 and 202 seconds respectively. Mefenamic acid was used as an internal standard (70). The oxidation of phenylbutazone was studied gas chromatographically on 2 m glass column, packed with 10 % 1,4-butandiol succinat on chromosorb W, 80-100 mesh at 130°C column temperature isotherm (71). It was shown that with alkaline peroxide and acidic permanganate valeric acid is obtained as oxidation product, while with alkaline permanganate butyric acid. 5 % OV 7 on Gas Chrom Q at 150°C was used for the estimation of intact phenylbutazone and of total degradation impurities in raw material and commercial dosage form with diphenyl phthalate as an internal standard (72). Phenylbutazone has been

determined in plasma without interference from the metabolites oxyphenbutazone and hydroxyphenylbutazone on 3% Apiezon L on Chromosorb W-HP, 80-100 mesh at 230°C isotherm (73). The drug was extracted from plasma after addition of 1 N HCl with n-heptane. About 96% of phenylbutazone was recovered from plasma through this method. Phenylbutazone and its metabolite oxyphenbutazone have been determined in plasma on a 5% OV 7 column by flash methylation with trimethylanilinium hydroxide using 1-(o-chlorophenyl)-1-(p-chlorophenyl)-2,2,2-trichloroethane as an internal standard (74). The method described is of sufficient sensitivity to determine plasma levels in humans after a 200 mg dose of phenylbutazone. Oxyphenbutazone and phenylbutazone have been determined in plasma and urine of dogs and horses by GLC on a 3% OV 210 on Gas Chrom Q column at 185°C isotherm (75). A GLC determination of phenylbutazone in human plasma down to 10 ng/ml is reported using 5% OV 17 on Chromosorb WHP column, 80-100 mesh and a ^{63}Ni -electron capture detector (76). A phenylbutazone analog 4-butyl-1,2-bis (p-tolyl)-3,5-pyrazolidine was chosen as an internal standard (76). Fluoranthene as the internal standard and N,O-bis (trimethylsilyl) trifluoroacetamide as the silylating agent have been used to determine phenylbutazone and its metabolites in human or rabbit plasma (77). Another sensitive and specific method for the detection of phenylbutazone in biological samples is its oxidation with permanganate to azobenzene and subsequent gas chromatographic determination on a 10% DC-200 on Gas Chrom Q, 80-100 mesh column with a flame ionization detector (78).

High Performance Liquid Chromatography

Phenylbutazone and oxyphenbutazone were determined in plasma on a Sil-X-adsorption column using a mobile phase of 0.002% glacial acetic acid and 10-23% tetrahydrofuran in n-hexane with a flow-rate of 1 ml/min at 35°C column temperature and 254 nm detection wavelength (79,80). 2,4-dinitrophenylhydrazine of benzaldehyde was used as an internal standard. Detection limit for phenylbutazone was found to be 0.2 $\mu\text{g/ml}$. Phenylbutazone and its metabolites oxyphenbutazone and γ -hydroxyphenylbutazone were determined in plasma and urine through HPLC on

Bondapack C₁₈ column using a mobile phase of methanol - 0.01M sodium acetate buffer (pH 4) in a linear gradient (50 to 100 % methanol at 5%/min with a flow-rate of 2.0 ml/min) and a detector wavelength of 254 nm (81). Calibration curves for all three compounds in the ranges of 0.5 - 5 µg/ml and 5 - 50 µg/ml were found to be linear. Plasma and urine samples were acidified with HCl, extracted with benzene - cyclohexane (1:1) and after evaporating the solvent residue was taken in methanol and chromatographed. Detection limit for phenylbutazone and its metabolites was 0.05 µg/ml (81). Same authors (82) have also reported the HPLC determination of phenylbutazone occurring as metabolite of anti-inflammatory agent suxibutazone in plasma and urine. C₁₈ Bondapack column was used with methanol-0.5M KH₂PO₄ as mobile phase in a linear gradient (from 0 to 100 % methanol at 8 %/min with a flow-rate of 2.0 ml/min) at 254 nm (82). Minute quantities of phenylbutazone (50-100 ng/ml) have been determined in plasma, urine, saliva and sweat of horses through HPLC on a C₁₈-Bondapack reversed-phase column with a mobile phase of 2 % glacial acetic in water-methanol (35+65) and a flow-rate of 2 ml/min. Detection was achieved at 240 nm (83). Another procedure for the determination of phenylbutazone and oxyphenbutazone is based on a microphase extraction from plasma and its subsequent determination through HPLC. A Lichrosorb RP 8 column and a mobile phase methanol-water-formic acid (75+250+0.6) were used at 25°C column temperature and 1.5 ml/min flow-rate. Detection was done at 238 nm. 0.5 µg compounds per ml plasma could be easily determined (84).

Polarography

The polarographic inactivity of phenylbutazone and oxyphenbutazone at the dropping mercury electrode led to the introduction of a derivatization procedure (85) which involves hydrolysis of the drug in a mixture of acetic and hydrochloric acids, diazotization, coupling with 2-naphthol and differential-pulse-polarography of the resulting azo-dyes. Phenylbutazone was determined in the range of 10⁻⁷M. Polarographic parameters used were forced drop time: 2s, pulse height: 50 mv and scan-rate of 2 mv/s. Two-electrode operation was used with a large surface calomel electrode as anode. The

polarogram was recorded between -0.6 to -0.8 V (85). Linear - sweep and differential-pulse voltammetric method for the determination of phenylbutazone and oxyphenbutazone in pharmaceutical dosage forms is also reported (86). The method is based on the electrochemical oxidation of both drugs at a glassy carbon electrode in 0.1 M sodium acetate-acetic acid in 98 % ethanol. An interrupted-sweep procedure is given for the determination of phenylbutazone in the presence of oxyphenbutazone (86).

9. Drug Metabolism and Pharmacokinetics

Several authors (87,88,89,90) have demonstrated that phenylbutazone underwent aromatic and side-chain oxidation, but the quantities in which the identified metabolites were present in human urine accounted for not more than a few percent of the dose. These amounts increased only insignificantly upon cleavage with β -glucuronidase. Unaltered phenylbutazone did not cover more than about 1 % (87,88). The elimination of the drug from blood is largely determined by biotransformation, since only a very small amount is removed as unchanged phenylbutazone by straight forward renal excretion (87,88). Phenylbutazone undergoes biotransformation in humans to oxyphenbutazone, γ -hydroxy, p- γ -dihydroxy and γ -oxo derivatives. The C-4 glucoronides of phenylbutazone and γ -hydroxyphenylbutazone are also known (91). The absorption from the gastrointestinal tract was found to be rapid and complete. After administration of ^{14}C -labelled phenylbutazone to a male volunteer the integrated concentration of unchanged phenylbutazone in plasma, as estimated from the area under the concentration curve (AUC) between 0 and 336 hours was 63% of that of total ^{14}C -substances (92). The corresponding AUCs of three specifically determined metabolites oxyphenbutazone, γ -hydroxyphenylbutazone and p- γ -dihydroxyphenylbutazone were 23 %, 2 % and 0.5 % respectively (92). A single oral dose was slowly excreted from the organism, since within 21 days only 88 % was recovered, 61 % from urine and 27 % from faeces (92). The sum of specifically measured metabolites (oxyphenbutazone, γ -hydroxyphenylbutazone, p- γ -dihydroxyphenylbutazone) and phenylbutazone in urine did not cover more than

about 10 %. About 40 % and 12 % of total urinary radioactivity was due to C-4-glucuronides of phenylbutazone and γ -hydroxyphenylbutazone respectively (92). Direct glucuronidation of phenylbutazone is the predominant biotransformation process (92). The time-course of the plasma concentration of unaltered drug is characterised by an early maximum of 36.0 $\mu\text{g/ml}$ at 3 hours and slow decay between 7 and 336 hours corresponding to an elimination half-life of 88 hours (92,93,94).

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SULFADIAZINE

Henry Stober and Wayne DeWitte

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1. Description

1.1 Name, Formula, Molecular Weight

Generic name - Sulfadiazine; Sulfapyrimidine; Pyrimal; Debenal (1).

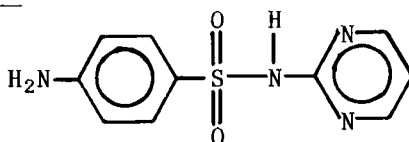
Nomenclature - The following nomenclature is used in Chemical Abstracts:

4-amino-N-2-pyrimidinyl-benzenesulfonamide [68-35-9]

Synonyms

N¹-2-Pyrimidinylsulfanilamide
p-Amino-N-(2-pyrimidyl)benzenesulfonamide

Structure



C₁₀H₁₀N₄O₂S

Molecular Weight: 250.27

1.2 Appearance, Color, Odor

White or slightly yellow powder. It is odorless or nearly so, and is stable in air but slowly darkens on exposure to light (2).

2.0 Physical Properties

2.1 Infrared Spectrum

The infrared spectrum (Figure 1) was determined for a KBr pellet preparation of Sulfadiazine USP (2) using a Perkin-Elmer Model 621 IR spectrophotometer. The assignments for important absorption bands are presented in Table I. Assignments were made using general sources (3) and literature references (4, 5).

2.2 Nuclear Magnetic Resonance (NMR) Spectrum

The NMR spectrum of sulfadiazine was obtained in DMSO-d₆ containing TMS as internal reference using a Perkin-Elmer Model R-24B NMR spectrometer (Figure 2). The spectral assignments are given in Table II (6).

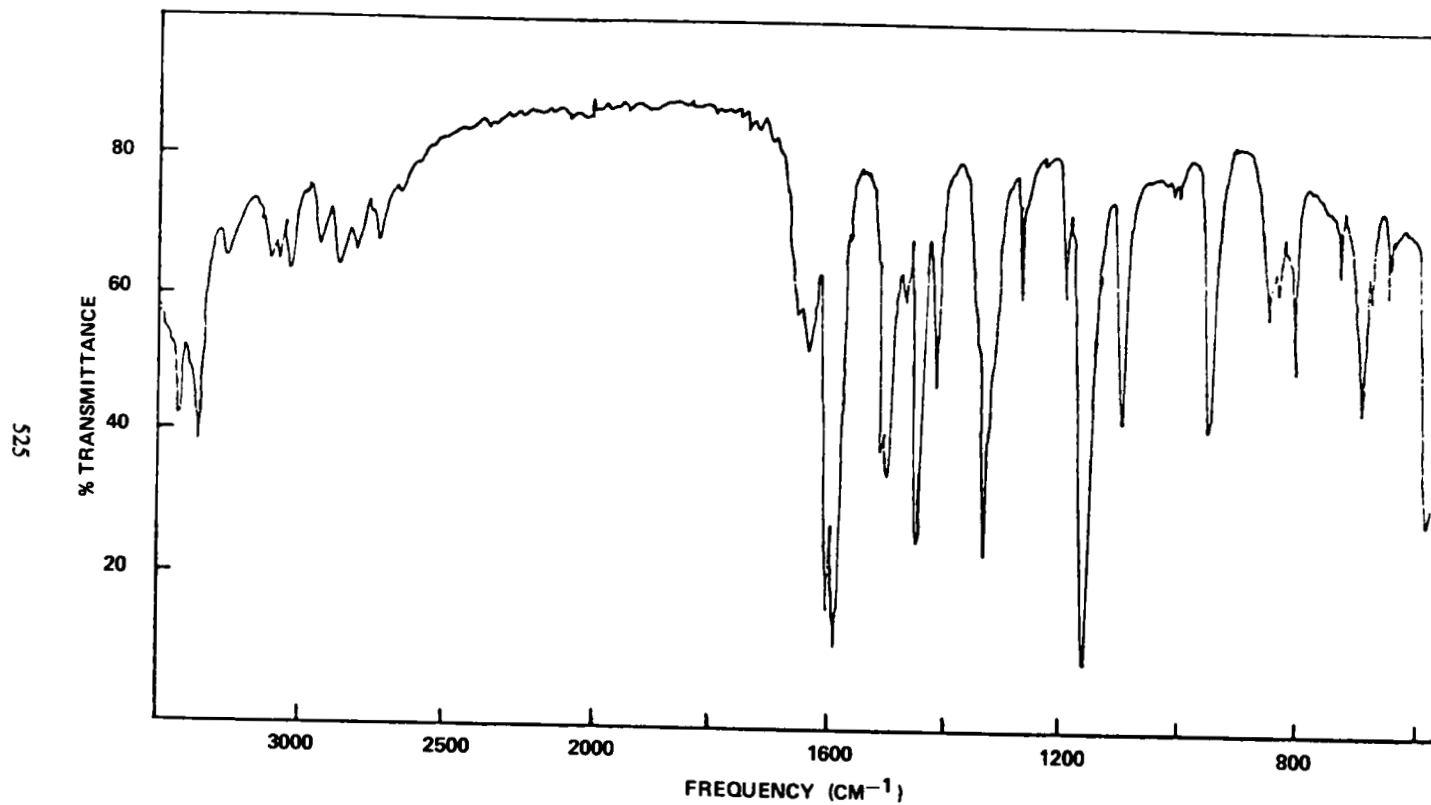


Fig. 1. IR Spectrum of Sulfadiazine

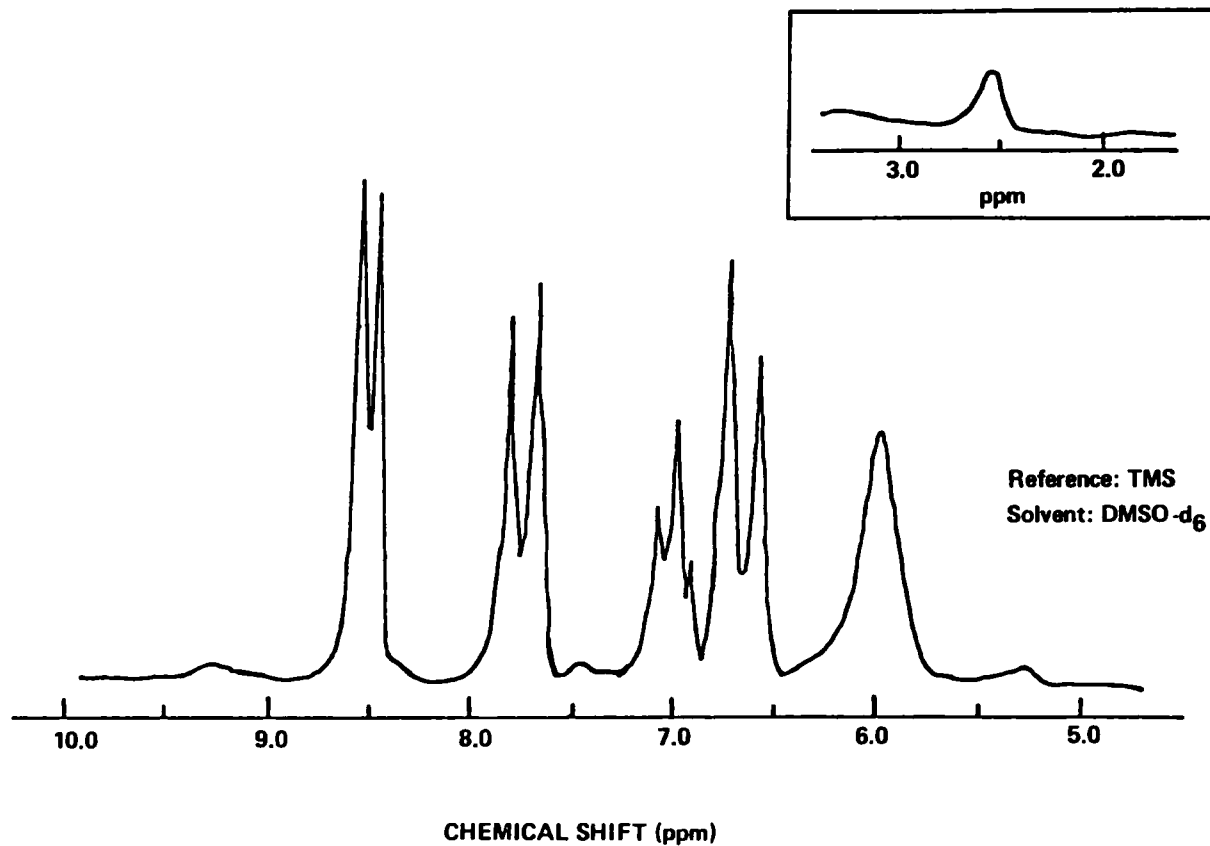


Fig. 2. NMR Spectrum of Sulfadiazine

Table I
Infrared Assignments for Sulfadiazine

Wavenumber (cm ⁻¹)	Assignment
3450 3410	N-H symmetric stretching
1650	NH ₂ deformation
1580 1490 1440 1410	Ring skeletal vibrations
1325	SO ₂ asymmetric stretching
1155	SO ₂ symmetric stretching

Table II
NMR Assignments for Sulfadiazine

δ (ppm)	Multiplicity	No. of Protons	Assignment
6.0	Singlet	2	
6.7	Doublet	2	
7.0	Triplet	1	
7.7	Doublet	2	
8.5	Doublet	2	
11.3	Singlet	1	SO ₂ NH

Turczan and Medwick (7) reported similar assignments for sulfadiazine and a classification scheme for the identification of sulfonamides based on their NMR spectra.

The natural abundance ^{13}C magnetic resonance spectrum of sulfadiazine has been reported by Chang and Floss (8).

2.3 Ultraviolet Spectra

The ultraviolet spectra for sulfadiazine were determined in 0.1M HCl, 0.1M NaOH and USP Simulated Intestinal Fluid, pH 7.5 (without enzyme). Solutions were scanned from 350 nm to 200 nm using a Cary 14 spectrophotometer (9). A summary of the data obtained is presented in Table III along with data obtained from the chemical literature.

Table III
Ultraviolet Spectral Values for Sulfadiazine

Solvent	λ_{max} (nm)	$A_{1\text{cm}}^{1\%}$
0.1M HCl	215	548
	242	579
0.1M NaOH	242	821
	254	794
USP Simulated Intestinal Fluid, pH 7.5 (without enzyme)	240	821
	254	794
Ethanol	270	844 (10)

The ultraviolet spectrum for sulfadiazine in 0.1M NaOH is presented in Figure 3.

2.4 Mass Spectrum

The mass spectrum of sulfadiazine (Figure 4) was obtained with a Kratos Model MS-25 mass spectrometer (11).

The mass spectra of sulfapyrimidines have been studied by Cambon and co-workers (12). For sulfadiazine, preferential fragmentation occurs to eliminate SO_2 resulting in the fragments observed at $m/e = 186$ and 185 . The m/e assignments for sulfadiazine are presented in Table IV.

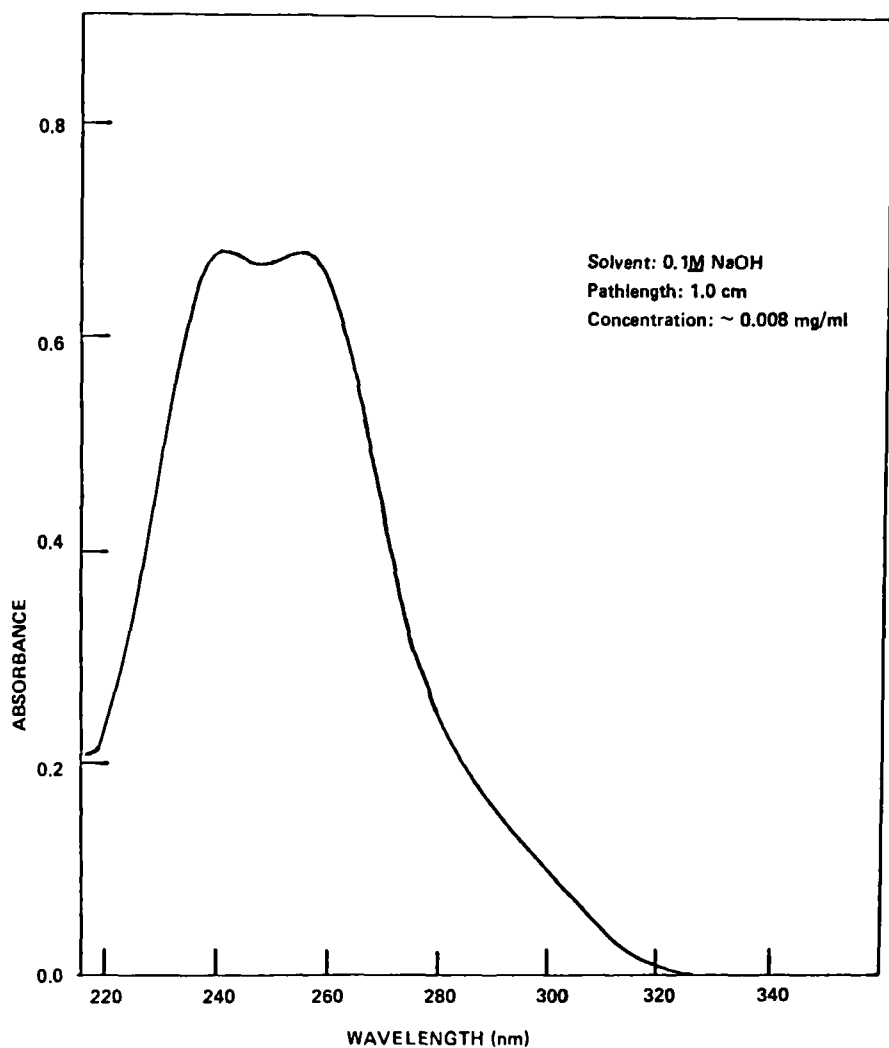


Fig. 3. UV Spectrum of Sulfadiazine

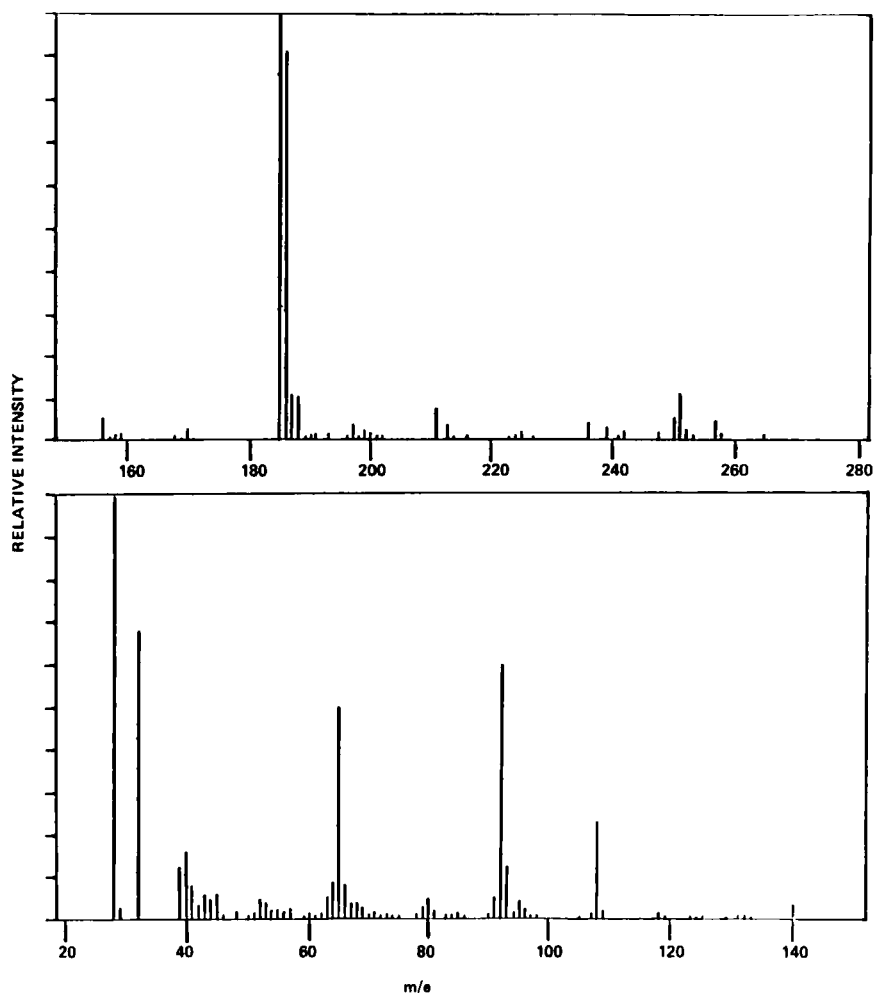
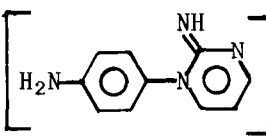
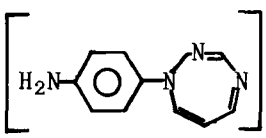
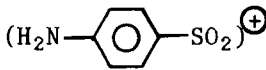
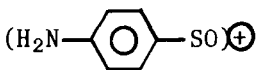
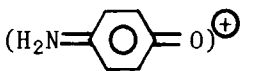




Fig. 4. Mass Spectrum of Sulfadiazine

Table IV
Mass Spectral Assignments for Sulfadiazine

m/e	Relative Intensity (%)	Assignment
251	0.5	$(M+1)^+$
250	0.2	M^+
186	90.9	 \rightleftharpoons 
185	100.0	Loss of H from m/e = 186
156	5.3	
140	3.2	
108	22.8	
92	59.4	
65	49.6	

2.5 Melting Range

The USP melting range specification for sulfadiazine is 251-254°C (2) using USP melting point procedure 1a (13). For a sample of Sulfadiazine USP, melting was observed from 253-254°C followed by decomposition (14).

2.6 Differential Thermal Analysis (DTA)

The thermogram for Sulfadiazine USP exhibits a sharp melting endotherm at about 260°C followed by decomposition (15). A typical thermogram is shown in Figure 5.

Sunwoo and Eisen (16) used DTA to determine the melting point and heat of fusion of sulfadiazine recrystallized from acetone. Values of 265.6°C and 7464 ± 180 cal/mole, respectively, were obtained.

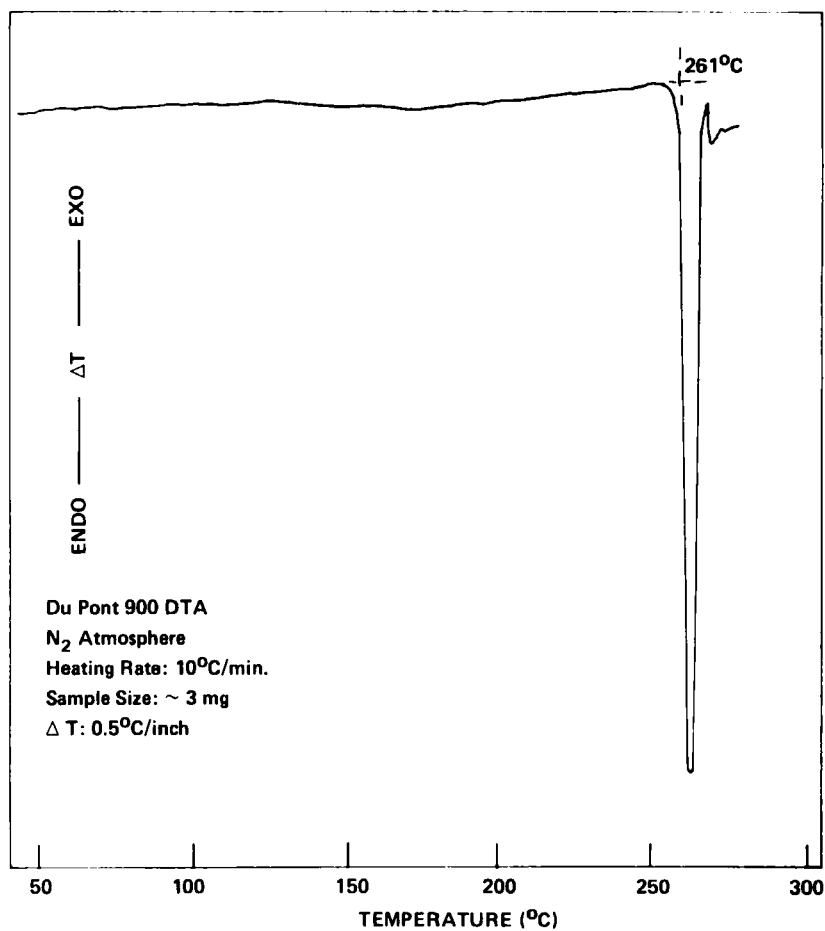


Fig. 5. DTA Thermogram of Sulfadiazine

2.7 Thermogravimetric Analysis (TGA)

The thermogravimetric behavior of Sulfadiazine USP in a N_2 atmosphere was determined with a Perkin-Elmer TGS-1 thermobalance at a scan rate of $10^\circ C/\text{minute}$ (17). For a 3 mg sample a weight loss of approximately 0.2% was observed from RT up to $220^\circ C$, followed by a gradually increasing weight loss attributed to decomposition at higher temperatures.

Cook and Hildebrand (18) have employed TGA for the identification of several sulfa drugs, including sulfadiazine. Scans were conducted at a rate of $5^\circ C/\text{min}$ up to about $800^\circ C$. At this temperature no residue of the sulfa drug was left in the sample pan. At lower temperatures SO_2 is suspected as the major product of pyrolysis.

2.8 Microscopy

Sulfadiazine USP powder is composed of transparent rod-like crystals which exhibit extinction and birefringence under crossed polars. The microscopic crystallographic properties of sulfadiazine reported in the literature have been summarized by Tillson and Eisenberg (19) and are presented in Table V.

Table V

Microscopic Crystallographic Properties of Sulfadiazine

Crystal System	N_α	N_β	N_γ	Optic Sign	2V	Ext.	Elong.	Habit
monoclinic	1.596	1.675	1.830	+	76°	P, i	\pm	lath shaped
unclassified	1.615	1.663	>1.734	-	--	P	\pm	rods

NOTE: The results reported for the unclassified system are presumed to represent intermediate data which are quite often obtained for some commercial samples and are probably related to hydration.

Sulfadiazine forms characteristic "burrs" of very fine needles when mixed with an acidic solution of gold chloride (20).

Treatment of sulfadiazine with a solution of potassium triiodide produces rosettes of blades or needles (10).

2.9 Polymorphism

An evaluation of polymorphism in sulfonamides has been conducted by Yang and Guillory (21). Sulfadiazine recrystallized from five different solvents did not exhibit polymorphism under the experimental conditions employed.

2.10 X-ray Powder Diffraction

The X-ray powder diffraction pattern of USP sulfadiazine is presented in Table VI (22). Strong lines are observed at 12.8, 21.3, 22.9 and 29.4 degrees 2θ for copper K_{α} radiation. The instrumental and experimental conditions are given below.

Instrumental Conditions:

G.E. XRD-5:	Spectrogoniometer
Generator:	30 kV, 13 mA
Tube Target:	Cu
Radiation:	Cu, Ni Filtered, $K_{\alpha} = 1.542\text{\AA}$
Optics:	1° Beam Slit
	MR Soller Slit
	0.2° Detector Slit
	3° Take-Off Angle
Goniometer:	Scan Rate: 2 degrees 2θ /minute (5°/inch)
Detection:	SPG-4 Detector
	Rate Meter, 2000 cps full scale
	Pulse Height Selection, $E_L = 0.4\text{V}$, $E_U = 1.5\text{V}$

Sample

Preparation: Sample was ground and back-packed into an aluminum sample holder (opening of 3 x 1 x 0.2 cm) without sieving. Compound acquires a high static electric charge upon sieving.

Table VI

X-ray Powder Diffraction Pattern of SulfadiazineMajor Lines

2θ Degrees*	d**	I/Io***
7.0	12.63	10
11.7	7.56	15
12.8	6.92	87
14.1	6.28	26
14.6(s)	6.07	6
16.2(s)	5.47	10
16.5	5.37	14
19.0	4.67	20
19.8	4.48	12
20.6(s)	4.31	5
21.3	4.17	81
22.9	3.88	100
24.5	3.63	7
25.9	3.44	25
27.4	3.26	22
28.2	3.16	18
29.4	3.04	52
30.5	2.93	3
33.2	2.70	3
34.6	2.59	5
36.0	2.50	16

*2θ degrees read to nearest 0.1 degrees

**Interplanar Distance: $d = \frac{n\lambda}{2 \sin \theta}$

***Relative Intensity in Per Cent Based on Strongest Signal.
Signals less than 3% relative intensity were excluded
based on 3 (S.D.) of noise level at 5° 2θ.

Under the experimental conditions employed, the relative intensities are subject to change due to variations in sample handling and particle size and are only included as a guide for identifying strong lines.

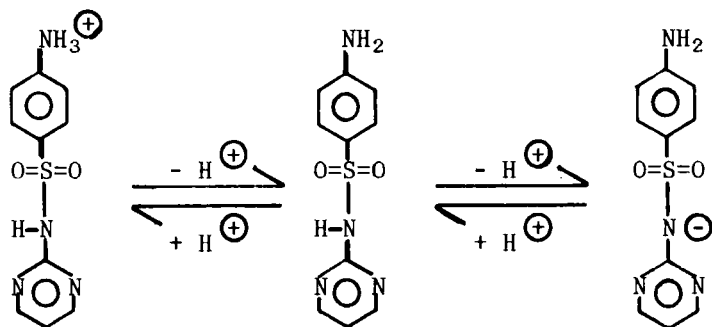
(s) Shoulder or peak poorly resolved from stronger signals.

2.11 Density and Contact Angle

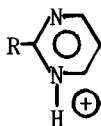
The density and contact angle, θ , of sulfadiazine have been reported by Lerk and co-workers (23). Using sulfadiazine conforming to the specifications of Pharmacopeia Nederlands, values of 1.48 g/cm³ and 28° (powder porosity of 0.168) were obtained.

2.12 Dissociation Constant:

Sulfadiazine is an ampholyte, and in aqueous solutions can exist in the protonated, neutral and anionic forms as illustrated below.



Salvesen and Schroder-Nielsen (24), using spectrophotometric techniques, reported a pKa value of 2.21 for the anilinium ion associated with sulfadiazine. These authors also stated that the pKa of the pyrimidinium ion



of 2-sulfanilamidopyrimidines is less than zero. Krebs and Speakman (25) used a solubility method to determine the acidity of the sulfonamide group. A pKa of 6.28 was obtained by these workers, while Willi and Meier (26) obtained a pKa of 6.35 using titrimetry. A summary of the pKa values reported in the literature for sulfadiazine is presented in Table VII.

Table VII
Reported pKa Values for Sulfadiazine

Group		Reference	Method
$-\text{NH}_3^+$	$\text{SO}_2-\text{N}-$ $\quad \quad $ $\quad \quad \text{H}$		
2.21	--	24	Spectrophotometry, 0.5M NaCl, T = 24°C
2.00	6.48	27	Experimental details not stated.
--	6.28	25	Solubility, $\mu = 0.1\text{M}$, T = 38°C
--	6.35	26	Titrimetry, $\mu = 0.1\text{M}$ (KCl), T = 20°C

2.13 Partition Coefficient

The partition coefficient of sulfadiazine was determined for the chloroform/0.1M HCl and chloroform/0.1M NaOH systems at ambient temperatures (~25°C). The partition coefficient presented in this monograph is defined as K_p where:

$$K_p = [\text{S organic}]/[\text{S aqueous}]$$

and [S] is the concentration of sulfadiazine in each phase.

<u>System</u>	<u>K_p</u>
CHCl ₃ /0.1M HCl	0.13
CHCl ₃ /0.1M NaOH	0.0005

Sulfadiazine reportedly is extracted from dilute aqueous acid solutions with ethyl ether (10).

2.14 Solubility

2.14.1 Equilibrium Solubility

The following equilibrium solubilities were determined at room temperature (~25°C) and 37°C for a sample of Sulfadiazine USP (28). An equilibration period of

about 24 hours was used for the 37°C condition (agitation provided by rotation at 15 rpm in a constant temperature bath) and 72 hours for the RT condition (agitation provided by a wrist action shaker). Analysis of the clear solution was performed by UV spectrophotometry.

<u>Solvent</u>	<u>Solubility (mg/ml)</u>	
	<u>RT</u>	<u>37°C</u>
0.1M HCl	0.61	0.75
Water	0.074	0.10
0.1M phosphate buffer (pH 7.4)	0.35	0.67

The solubility of sulfadiazine in water has been reported in the literature (29) as approximately 1 g in 1300 ml (0.08 mg/ml) at RT and 1 g in 60 ml (16.7 mg/ml) of boiling water. Sulfadiazine is also reported as being sparingly soluble in alcohol and acetone, freely soluble in dilute mineral acids and solutions of potassium and sodium hydroxides (2).

The solubility of sulfadiazine in water and biological fluids at 37°C was recently reported in the Federal Register (30).

<u>Solvent</u>	<u>Solubility (mg/ml) @ 37°C</u>
Water (pH 5.5)	0.14
Serum	1.60
Urine (pH 5.5)	0.18
Urine (pH 8.0)	4.50

The solubility of sulfadiazine in several normal alcohols was reported by Mauger and co-workers (31). The highest solubility occurred in methanol. The Hildebrand solubility parameters of sulfadiazine and other selected sulfonamides were determined by Sunwoo and Eisen in several alcohol-water-glycol systems (16). Elworthy and Worthington determined the solubility of sulfadiazine in water, dimethyl-formamide and a range of mixtures of these solvents (32).

2.14.2 Dissolution Rate

The poor solubility of sulfa drugs, such as sulfadiazine, in water was a factor in prompting the Food and Drug Administration to establish bioequivalence require-

ments for dosage forms of these compounds. For oral solid dosage forms of sulfadiazine, twelve tablets must be evaluated using the USP Apparatus II, 50 rpm, 37°C and 0.1M HCl as the dissolution medium. Specifications of 50% released in 30 minutes and 80% released in 60 minutes were indicated (30).

The intrinsic dissolution rate of sulfadiazine in water was measured by Nogami and co-workers (33) using the rotating disk method. The dissolution of sulfadiazine under these conditions was found to be in accord with the Noyes-Nernst equation concerning transport-controlled processes. In a subsequent publication these authors used the same technique to study the dissolution of sulfadiazine in different aqueous solutions (34).

3. Synthesis

The synthesis of sulfadiazine has been described by Roblin (35). Northey (36) has described the manufacturing procedure used for sulfadiazine. The synthetic reactions consist of condensing 2-aminopyrimidine with *p*-acetamidobenzenesulfonyl chloride, followed by hydrolysis of the N⁴-acetyl group with sodium hydroxide. Scheme I depicts the reactions used by Roblin and co-workers.

4. Inorganic Compounds

4.1 Sodium Salt

The sodium salt of sulfadiazine is listed in the USP XIX (37) and is employed medically when parenteral therapy is indicated. Exposure of this compound to humid air results in the gradual absorption of carbon dioxide with the corresponding liberation of sulfadiazine. About 1 gram of sulfadiazine sodium dissolves in 2 ml of water.

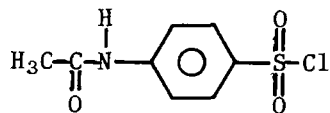
4.2 Other Inorganic Compounds

Silver sulfadiazine has found use as an anti-bacterial agent in the treatment of extensive burns. Spectroscopic data for silver sulfadiazine have been reported in the literature (38, 39). Bult and Klasen (40) investigated the structure of silver sulfadiazine and indicate that the silver coordinates with both the sulfonamide group and the nitrogens of the 2-aminopyrimidine substituent. A 1:1 complex is formed.

Other metallic compounds of sulfadiazine reported in the literature include those of the divalent metals Zn, Cd, Hg, Ni and Mn as well as trivalent Fe (41).

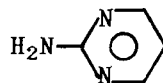
Scheme I

Synthesis of Sulfadiazine

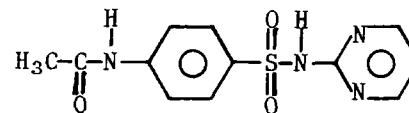


p-Acetamidobenzene-sulfonyl chloride

+

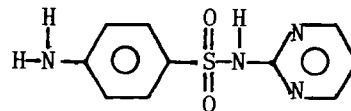
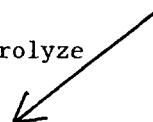


2-aminopyrimidine



N⁴-Acetylsulfadiazine

hydrolyze



Sulfadiazine

5. Chemical Stability

Sulfadiazine is stable in the solid state upon exposure to air, humidity and temperature up to 100°C for two weeks (42). It darkens upon exposure to light (2). Upon pyrolysis sulfadiazine yields 2-aminopyrimidine (43) and sulfur dioxide (18). In solution sulfadiazine undergoes acid-catalyzed hydrolysis via two pathways. The first pathway yields sulfanilic acid and 2-aminopyrimidine (43, 44, 45), whereas the second pathway produces sulfanilamide and 2-hydroxypyrimidine (43, 46). The kinetics of the autoxidation of sulfadiazine in solution at pH 2.0-9.2 have been reported by Zajac (47).

6. Methods of Analysis

6.1 Elemental Analysis

The results from an elemental analysis of sulfadiazine are listed below (48).

Elemental Analysis of Sulfadiazine

<u>Element</u>	<u>% Theory</u>	<u>% Found</u>
C	47.99	47.80
H	4.03	3.92
N	22.39	22.23

6.2. Volumetric Methods

The routine assay of sulfonamides can be accomplished using any of a variety of titrimetric methods. The most widely used assay procedure is titration with sodium nitrite solution to determine the aromatic amine function (49).

Several sulfonamides, including sulfadiazine, were determined by titration with KBrO_3 , the end point being indicated by a biamperometric system with platinum-graphite electrodes (50).

Agarwal, et al. (51) have described a spectrophotometric titration procedure for sulfadiazine and other sulfonamides. The drug is dissolved in a mixture of hydrochloric acid and glacial acetic acid and is then titrated with a 0.1N bromate-bromide mixture. The endpoint is determined by monitoring the absorbance at 345 nm spectrophotometrically.

Greenhow and Spencer (52) have reported a catalytic thermometric titration for sulfonamides. The drug is dissolved in dimethylformamide and is titrated with tetra-*n*-butylammonium hydroxide. The endpoint is detected by monitoring the heat evolved from the alkali-catalysed anionic polymerization of acrylonitrile, which is used as the indicator.

6.3 Spectrophotometric Methods

The Bratton-Marshall reaction (53), in which sulfadiazine is diazotized and then coupled with *N*-(1-naphthyl)-ethylenediamine dihydrochloride to give a colored product, is one of the most commonly used tests for sulfonamides.

Davis and co-workers (54) report a method for determination of sulfonamides following formation of an indophenol dye and measurement of absorbance at 725 nm.

A method specific for sulfadiazine in the presence of other sulfanilamidopyrimidines has been reported (55). The drug is reacted with 2-thiobarbituric acid and is determined by measurement of the absorbance at 305 nm.

Sulfadiazine present in tablets, solutions for injection, eye drops, blood or urine may be determined by diazotization, followed by coupling with phloroglucinol under acid conditions. The resultant yellow color is monitored at 415 nm (56).

6.4 Column Chromatography

A method for the separation and quantitation of sulfonamides has been reported by Rader (57). The procedure is based on formation of ion pairs between the sulfonamides and tetrabutylammonium ion, followed by separation on partition chromatographic columns. The separated sulfonamides are then determined by ultraviolet spectrophotometry.

Another method for separation of sulfonamides, including sulfadiazine, involves use of three columns. Celite support material is coated with phosphate buffer (pH 1.7 or 7.8) or borate buffer (pH 8.7), the sample applied and eluted with chloroform. The separated sulfonamides are then determined colorimetrically following derivatization by the Bratton-Marshall reaction (58).

6.5 High Performance Liquid Chromatography

Cobb and Hill (59) have reported a normal phase high performance liquid chromatographic separation of sulfonamides. Sulfadiazine was separated from a number of other sulfonamides using a silica column and a mobile phase containing cyclohexane (85.7%), anhydrous ethanol (11.4%) and glacial acetic acid (2.9%).

Other separation methods for sulfadiazine in the presence of other sulfonamides and of other antibacterial drugs have been reported, utilizing a wide range of stationary phases. These include reverse phase octadecylsilane packing (60), reverse phase octylsilane (61), ion pairing chromatography (62), cation exchange (63), and anion exchange (64).

Sulfadiazine has been separated from a number of other sulfonamides by taking advantage of the differing complexation reactions with cadmium(II) and zinc(II) ions (65). The stationary phases were either *n*-propylethylenediamine bonded to silica, or commercially prepared C₁₈ and C₈ columns loaded with 4-dodecyl-diethylenetriamine (in the mobile phase). The effects of metal ion concentration and type are discussed.

6.6 Gas Chromatography

Sulfapyrimidines have been analyzed by gas chromatography following acid hydrolysis to give sulfanilic acid and the respective aminopyrimidines. The aminopyrimidine is then analyzed using a column containing 5% SE-30 and 5% Carbowax 20M on Chromosorb W at a column temperature of 150°C (66).

Sulfadiazine and its principal metabolite, N⁴-acetylsulfadiazine, in plasma or urine, have been analyzed by gas chromatography with electron capture detection following derivatization with azomethane (67).

6.7 Paper Chromatography

Sulfadiazine and other sulfonamides have been analyzed by paper chromatography using several developing solvents and detection methods (68). The visualization method most suitable for sulfadiazine was reported to be reaction with Ehrlich reagent (*p*-dimethylaminobenzaldehyde in 1M HCl). The chromatography was performed on Whatman No. 1 filter paper. The R_f values for sulfadiazine were 0.82, 0.76, 0.45, 0.00, 0.24, and 0.87 in solvent systems A-F, respectively.

The solvent systems used were:

- A. Methyl isobutyl ketone-formic acid-water (10 parts ketone saturated with 1 part 4% formic acid).
- B. Chloroform-methanol-formic acid-water (10 parts chloroform saturated with a mixture of 1 part methanol and 1 part 4% formic acid).
- C. Benzene-methyl ethyl ketone-formic acid-water (a mixture of 9 parts benzene and 1 part ketone saturated with 1 part 2% formic acid).
- D. Benzene-formic acid-water (10 parts benzene saturated with 1 part 2% formic acid).
- E. Methyl ethyl ketone-diethylamine-water (921:2:77).
- F. Methyl ethyl ketone-acetone-formic acid-water (40:2:1:6).

6.8 Thin Layer Chromatography

Sulfadiazine can be determined in mixtures of sulfonamides using a thin layer chromatographic separation followed by ultraviolet spectrophotometric analysis (69). The samples are spotted on fluorescent silica gel H plates and developed in chloroform-methanol (88:12). The spots of interest are scraped off the plate after delineation under UV light and extracted with 1M NaOH. The absorbances of the centrifuged extracts are read with a recording spectrophotometer.

Walash and Agarwal (70) report a separation of sulfadiazine from other sulfonamides. Sulfadiazine has an R_f of 0.56 using silica gel plates developed with a mixture of ethyl acetate-methanol (9:1). The spot can be located using any of the following reagents:

- (1) saturated copper acetate in methanol (brown spot)
- (2) saturated copper acetate in acetone (brown)
- (3) 5% copper sulfate in water (brown)
- (4) 2% ceric sulfate in water with 5 ml of concentrated H_2SO_4 (yellow).

The chromatographic behavior of a variety of sulfonamides including sulfadiazine has also been studied on strong and weak cation and anion exchange TLC plates with several matrices using both aqueous and nonaqueous solvent systems (71).

Clarke and Humphreys (72) describe methods for identification of twenty-five sulfonamides using silica gel G plates coated with NaOH, KHSO₄, H₂O and NaOH and solvent systems 1-4, respectively. The spots are located using copper sulfate, p-dimethylaminobenzaldehyde, N-(1-naphthyl)-ethylenediamine·2HCl or fluorescein.

- (1) chloroform-methanol (4:1)
- (2) chloroform-carbon tetrachloride-methanol (7:2:1)
- (3) ethylacetate-methanol (9:1)
- (4) acetone-methanol (4:1)

The separation of sulfadiazine from two of its known hydrolysis products, sulfanilamide and sulfanilic acid, has been reported using silanized silica gel layers impregnated with triethanolamine dodecylbenzenesulfonate or N-dodecylpyridinium chloride (73). Their behavior and that of other sulfonamides was also studied on layers not impregnated with detergents.

Cieri (74) describes a method for the detection of sulfadiazine and other sulfonamides in animal feeds. The sulfonamides are extracted from feed samples with alcohol or acetone and are cleaned up by a Celite column chromatographic technique. The eluate is spotted on an Adsorbosil-1 TLC plate, which is developed in chloroform-methanol (95:5) and then sprayed with an alcoholic solution of p-dimethylaminobenzaldehyde.

7. Pharmacology

Sulfadiazine is used for the cure of infections originating from susceptible Gram-positive bacteria. It is absorbed readily from the gastrointestinal tract to yield reproducible blood levels. The solubility of sulfadiazine in urine makes it suitable for the treatment of E. coli infections in the urinary tract. The systemic toxicity of sulfadiazine is low, and relatively few side effects are associated with its use (75, 76). The chronic toxicity of sulfadiazine has been reported by Schmidt (77).

The metabolism of sulfadiazine in humans involves acetylation (in the liver), oxidation and hydrolysis. In addition to unchanged sulfadiazine, which accounted for over 50% of the products excreted, Uno and Sehine (78, 79) identified the following metabolites in human urine:

N⁴-acetylsulfadiazine, sulfadiazine
N⁴-glucuronide, sulfadiazine sulfonate and
sulfanilamide.

The analytical method employed by these authors involved separation of the metabolites by paper chromatography followed by elution of each component and analysis using Ehrlichs reagent. The Bratton-Marshall reaction (80) and gas chromatography with electron capture detection (67) have also been employed for the analysis of sulfadiazine in biological fluids.

The protein binding of sulfadiazine and other sulfa drugs has been investigated by Hsu and co-workers (81). They found that binding affinity increased with the number of methyl substituents in the 2-pyrimidine ring.

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LEVARTERENOL BITARTRATE

Terry D. Wilson

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1. Foreword

Levarterenol bitartrate is a strong α and β_1 adrenergic agent having peripheral vasoconstriction and coronary artery dilatation properties. It is indicated in the treatment of acute hypotensive states and cardiac arrest. Administration is done I.V. in a 5% dextrose solution in distilled water or saline at a dose of 1-10 μ g per minute.¹⁻⁴ The present supplement follows the original Analytical Profiles review.⁵

2. Description

2.1 Nomenclature

Levarterenol Bitartrate
 Levophed® Bitartrate
 (R)-4-(2-amino-1-hydroxyethyl)-1,2-benzenediol [R-(R*,R*)] -2,3-dihydroxybutanedioate (1:1) salt, monohydrate

l-Norepinephrine Bitartrate

2.2 Formula

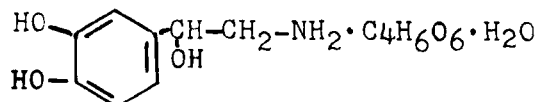
$C_8H_{11}NO_3 \cdot C_4H_6O_6 \cdot H_2O$

2.3 Molecular Weight

hydrate 337.28

anhydride 319.27

2.4 Structure



2.5 CA Registry Number

Hydrate [69815-49-2]

Anhydride [51-40-1]

3. Physical Properties

3.1 Nuclear Magnetic Resonance

The 1H -nuclear magnetic resonance spectrum of levarterenol bitartrate monohydrate is shown in Figure 1. The spectrum was taken from a 10% solution in D_2O on a Varian HA 100 spectrometer with a TMS external standard. Assignments of chemical shifts are shown in Table I.

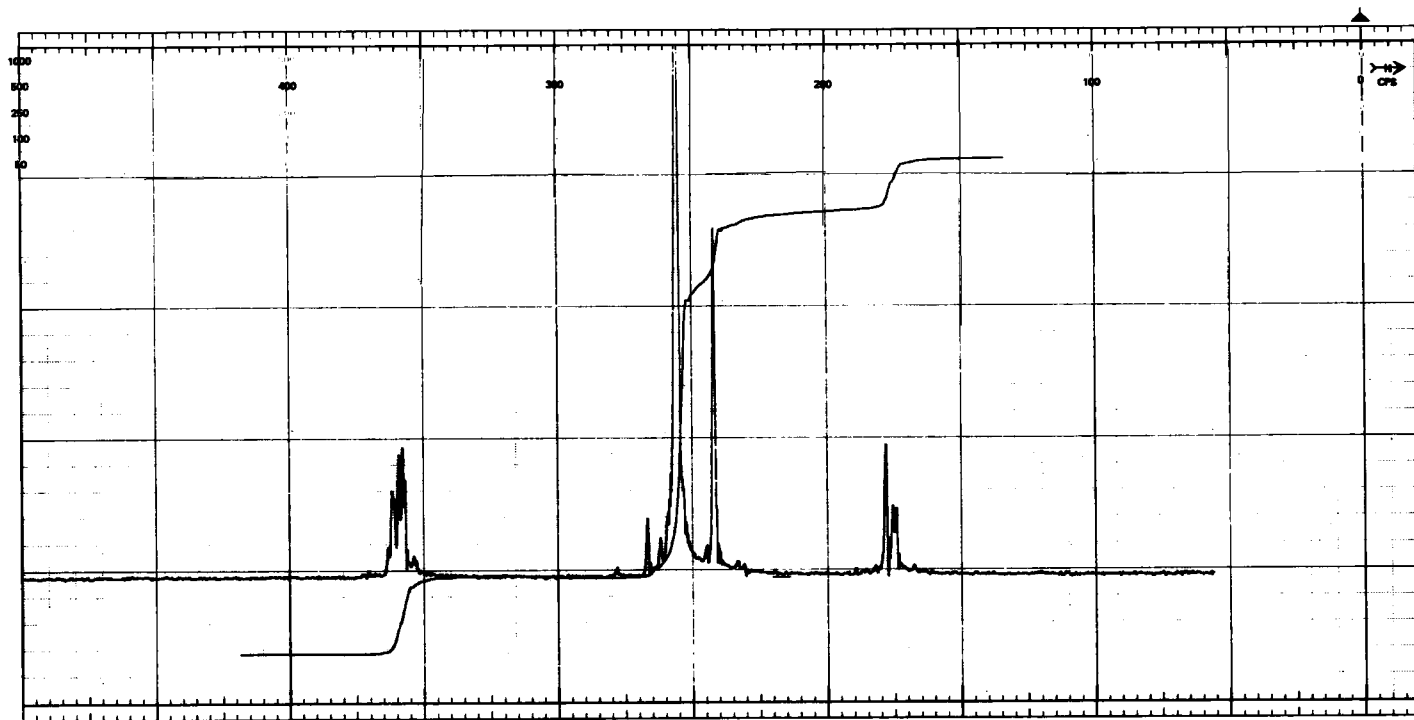


Figure 1. ^1H nuclear magnetic resonance spectrum of levarterenol bitartrate monohydrate.

Table I
 ^1H n.m.r. Chemical Shift Data for
Levarterenol Bitartrate

<u>Chemical Shift</u> <u>ppm (TMS)</u>	<u>No. H</u>	<u>Assignments</u>
7.02-7.3	3	aromatic H
5.11	11	OHx7, NH, H ₂ O (exchange) OCH
4.84	2	OCH
3.48-3.6	2	NCH ₂

These agree with previous results of Reisch et al^{6,7} who also used TMS as external standard. It was not possible however to convert the primary amine of levarterenol to a TMS derivative using HMDS for purposes of an n.m.r. spectrum.⁸ Granot expressed chemical shift data as τ values relative to a p-dioxane internal standard for levarterenol solutions.⁹ He was able to show evidence for intermolecular self-association by varying concentrations in D₂O and DMSO solvents. Upfield and downfield shifts were found respectively for the phenyl protons with increasing concentrations.¹⁰

3.2 Mass Spectrum

A mass spectrum of levarterenol base is shown in Figure 2 taken on a HP 5980A. Extensive fragmentation of the bitartrate salt in the ion source prevented acquisition of a useful spectrum. A weak molecular ion (M^+) can be seen at m/e 169. The base peak at m/e 139 can be attributed to $M^+ - \text{CH}_2\text{NH}_2$. Other peaks seen are m/e 151, $M^+ - \text{H}_2\text{O}$; m/e 111, $M^+ - \text{CHOHCH}_2\text{NH}_2$ and m/e 93, $M^+ - \text{H}_2\text{O} - \text{CHOHCH}_2\text{NH}_2$.

Mass spectrometry combined with gas chromatography (GC-MS) on levarterenol has been reported by several authors. These procedures involve derivitization and monitoring specific m/e peaks commonly employing multiple ion detectors (MID). Pentafluoropropionyl derivatives have been prepared^{11,12,13,14} and the ions of m/e 753, molecular ion; 577, $M^+ - \text{CH}_2\text{NHCOC}_2\text{F}_5$; 549, $M^+ - \text{CH}_2\text{NHCOC}_2\text{F}_5 -$

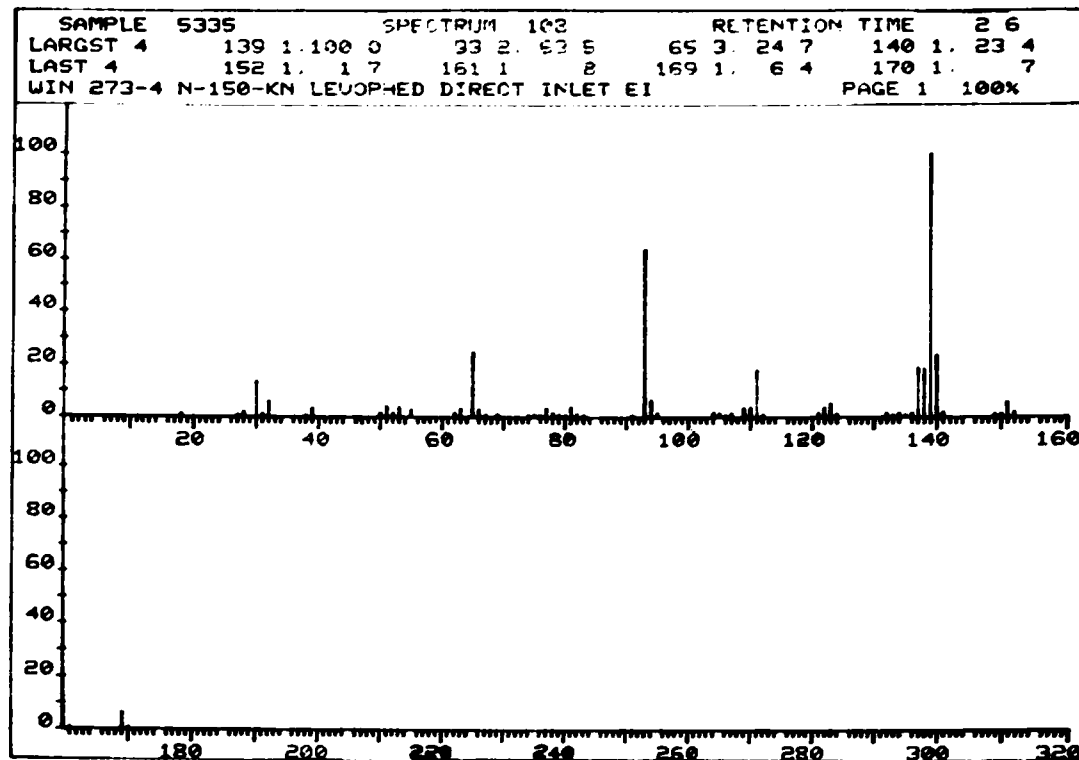


Fig. 2. Mass spectrum of levarterenol base.

CO and 176, $^+\text{CH}_2\text{NHCOC}_2\text{F}_5$ have been monitored. N-pentafluorobenzyl-O-trimethylsilyl derivitization has also been carried out with the following ions resulting: 563, molecular ion; 548, $\text{M}^+ - \text{CH}_3$; 355, $\text{M}^+ - \text{CH}_2\text{N}=\text{CHC}_6\text{F}_5$; 208, $\text{CH}_2=\text{N}^+=\text{CHC}_6\text{F}_5$ and 179, C_7F_5^+ .

3.3 Dissociation Constants

No general agreement has been reached on the values of dissociation constants for levarter-enol. The results of several reports are shown in Table II.

Table II

Dissociation Constants

pK_{a1}	pK_{a2}	pK_{a3}	reference
8.72	9.72	12	4
9.3	10.3	13	9
8.57	9.73	11.13	16

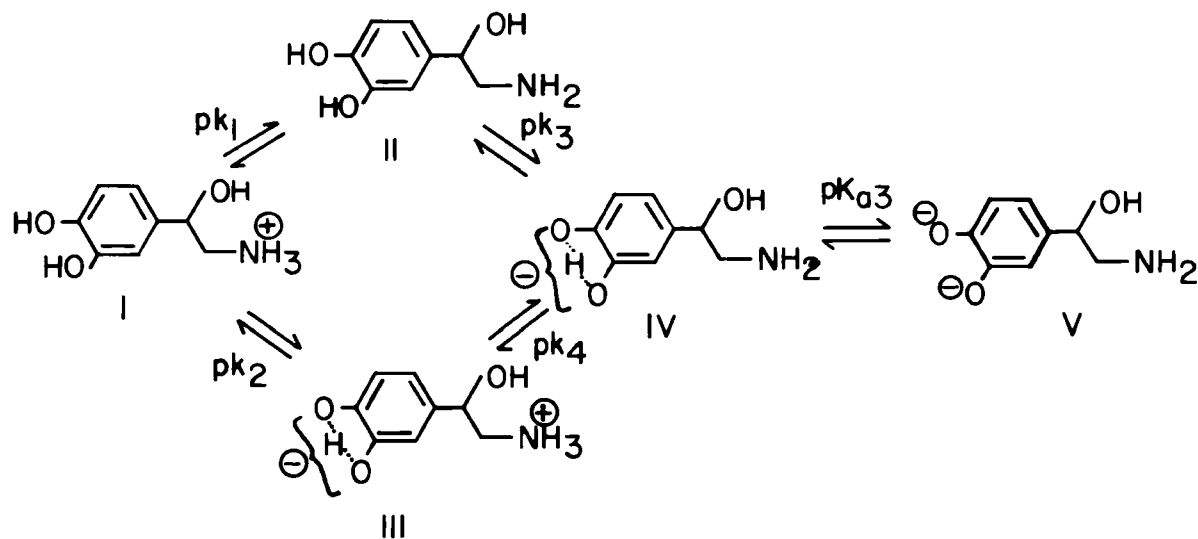
While pK_{a3} results from the ionization of the second phenolic group, pK_{a1} and pK_{a2} as determined by titration procedures are assigned to the first phenolic and the ammonium ion or vice versa. It has been pointed out⁹ that the ionization of these two groups does not occur independently and can be thought of as simultaneous reactions as pictured in Scheme 1. I is the cationic form, II is the neutral, III the zwitterionic, IV the monoanionic and V the dianionic form.

The correct statements for the relation between the macro- and micro-ionization constants are:

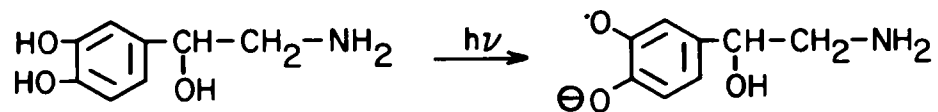
$$\text{K}_{\text{a1}} = \text{k}_1 + \text{k}_2$$

$$\frac{1}{\text{K}_{\text{a2}}} = \frac{1}{\text{k}_3} + \frac{1}{\text{k}_4}$$

SCHEME 1



SCHEME 2



Ganellin was able to derive a tautomeric equilibrium constant, K_t , from the microconstants using the relation: $K_t = \text{antilog}(pk_1 - pk_2)$. This gave the ratio of the zwitterion to the uncharged species with a value of 1.8 at 25° C in water.¹⁷

3.4 Electron Spin Resonance

A high resolution E.S.R. spectrum has been obtained for levarterenol semiquinone anion-radical and was described as identical to that of epinephrine.¹⁸ The photooxidation giving rise to the anion-radical is pictured in Scheme 2.

E.S.R. parameters for the anion-radical are: $A(5-H) = 3.58 \text{ G}$, $A(7-H) = 3.03 \text{ G}$, $A(6-H) = 0.96 \text{ G}$, $A(3-H) = 0.48 \text{ G}$ and $g = 2.0044$.

4. Stability

4.1 Oxidation

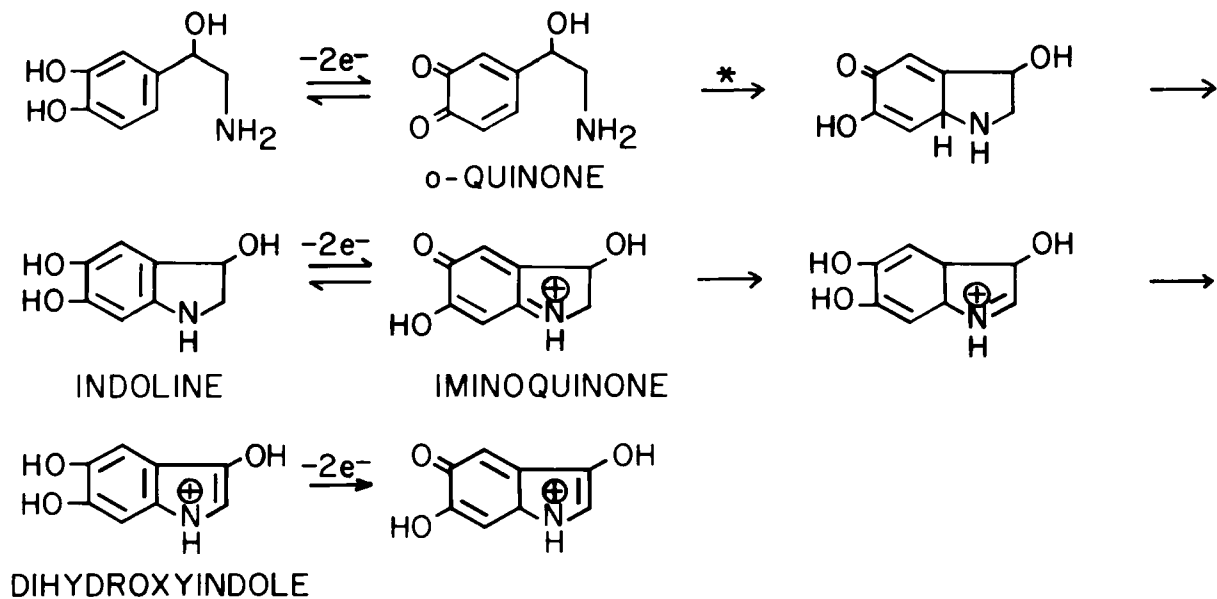
The oxidation of levarterenol has been shown to proceed via pH-dependant pathways including intramolecular cyclization^{19,20,21} and external nucleophilic attack²⁰ as seen in Schemes 3a and 3b. These reactions were followed by cyclic voltammetry.

Whereas the cyclization reaction has an overall rate constant of 0.066 sec^{-1} at pH 6.0¹⁹, the nucleophilic reaction rate varies with the nucleophile from 0.22 to 2100 sec^{-1} for the amino acids cystine to cysteine respectively at pH 7.4.

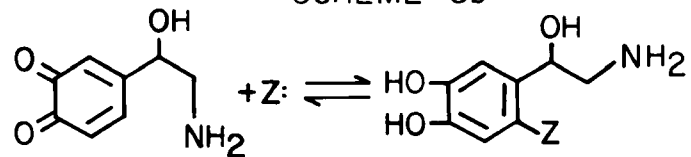
4.2 Dosage Form Stability

Stabilization of levarterenol in solution dosage forms has been attained by addition of antioxidants in various combinations. These include sodium bisulfite and the formula shown in Table III. Here ascorbic acid is the principle antioxidant.²²

SCHEME 3a



SCHEME 3b



* RATE DETERMINING STEP

Table III
Levarterenol Solution Dosage Form

	<u>% by Wt.</u>
Norepinephrine	1.00
Boric acid	1.50
Ascorbic acid	0.50
N-acetyl-L-cysteine	0.50
Sodium carbonate q.s. pH 6.0	
Purified water, q.s.	

5. Methods of Analysis

5.1 Colorimetric Titration

The U.S.P. assay for norepinephrine bitartrate is carried out by titrating a solution made by adding approximately 500 mg norepinephrine bitartrate to 20 ml glacial acetic acid. The titrant used is 0.1 N perchloric acid. When the crystal violet endpoint is reached, each ml of 0.1 N perchloric acid is equivalent to 31.93 mg of norepinephrine bitartrate anhydride.²³

5.2 UV Spectrophotometric

Norepinephrine bitartrate in the injectable dosage form is determined according to the U.S.P. by a spectral method. Absorbance of the sample eluted from a siliceous earth column is compared to that of the standard at 40 µg/ml in 1 in 350 dilute sulfuric acid. The absorbance is determined at λ_{max} of 278 nm and also at two minima: 250 and 300 nm to correct for baseline.²⁴

The presence of sodium sulfite as anti-oxidant in levarterenol solution dosage forms was shown to cause interference in the UV assay unless it was carried out at pH 7.0.²⁵

5.3 Fluorescence

Quantitation of levarterenol by fluorescence measurement provides a sensitive means of analysis. Reactions producing fluorescent products include oxidation to an ethylenediamine condensation product (EDA) and a trihydroxyindole

product (THI) shown in Schemes 4a and 4b respectively.²⁶

Early descriptions of these methods are attributed to: Weil-Malherbe²⁷ and Anton and Sayre.²⁸ Elution from a cation exchange resin at pH 6 is used with²⁹ or without³⁰ prior aluminum oxide adsorption with hexacyanoferrate(III) oxidation in the former method. The THI method was originally developed using the same oxidizing agent but has been modified to include an iodine/iodide oxidation^{31,32,33} and automation.³⁴

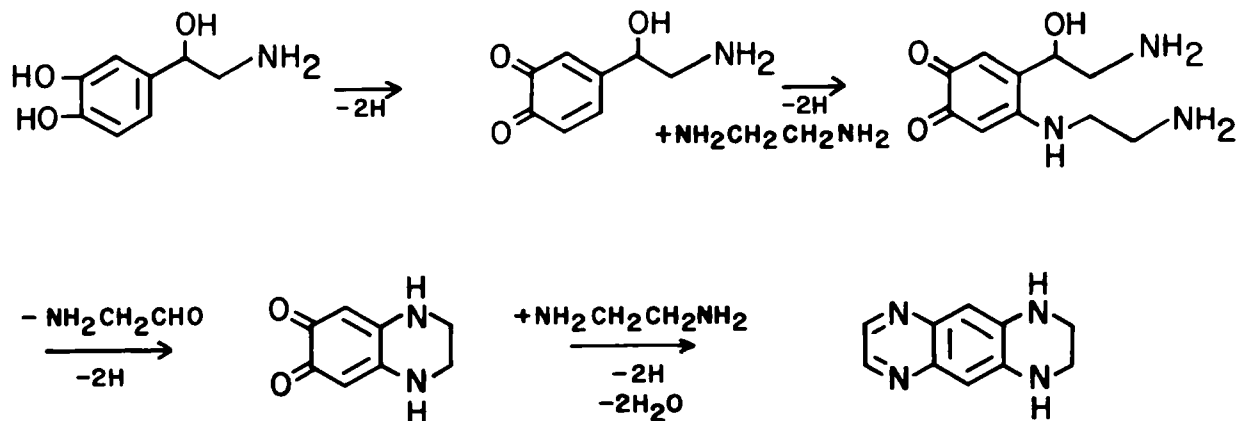
5.4 Radioenzymatic

Sensitive methods for levarterenol have been developed utilizing an enzymatic transformation of the compound to a radioactive product which is measured by liquid scintillation. Typical single isotope derivitization techniques are illustrated in Schemes Va and Vb. These reactions are catalyzed by the enzymes catechol-O-methyltransferase (COMT)³⁵ and phenethanolamine-N-methyltransferase (PNMT)³⁶ respectively. These give rise to tritiated products when an ³H-labeled coenzyme (S-adenosylmethionine) is present. Early methods were very tedious and time consuming requiring up to four days per sample³⁵ however they have been simplified and commercialized.³⁷ Products of the enzymatic reaction were separated by TLC after extraction with toluene:i-amyl alcohol (3:2), oxidized to vanillin with periodate and counted by liquid scintillation in the original methods.^{35,38,39} Later methods however eliminated the oxidation step,^{40,41,42} while another modification was a double isotope derivitization which allowed for concomitant percentage recovery determination.³⁹

5.5 Immunoassay

An immunoassay method was used by Miwa et al⁷ to measure norepinephrine. N-maleylnorepinephrine was conjugated to bovine serum albumin by a Mannich reaction. Following removal of the maleyl group, immunization of rabbits gave antiserum specific for the conjugate.

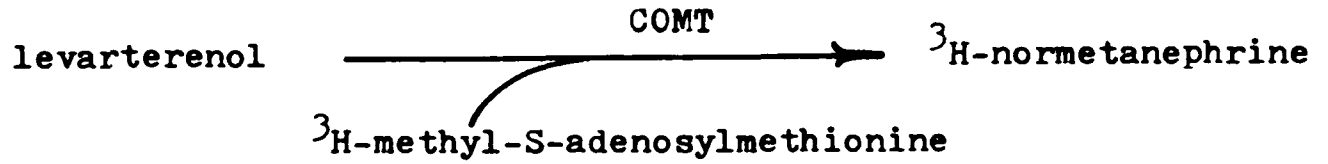
SCHEME 4a



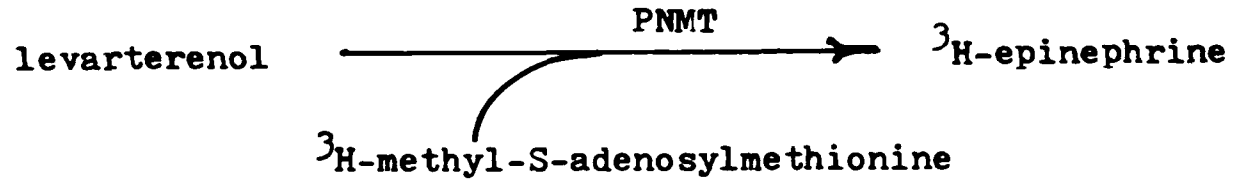
SCHEME 4b



SCHEME 5a



SCHEME 5b



5.6 Chromatography

5.61 Thin Layer and Paper Chromatography

Thin layer and paper chromatographic systems for the separation and determination of levarterenol are listed in Table IV.

5.62 Gas Chromatography

Specific and sensitive methods for levarterenol have been developed using gas chromatography. Table V includes information on column material, derivitization, detection and detection limits. Reference 48 contains a review of previous g.c. methods.

5.63 High-Performance Liquid Chromatography

Developments in the field of high-performance liquid chromatography have supplemented former methods for levarterenol analysis, especially in biological samples where picogram levels are routinely encountered. Ease, low cost and rapidity of operation in addition to sensitivity and specificity are afforded by these methods. This section is arranged according to detection methods with a tabulation of column, mobile phase and detection limit information in Table VI.

5.631 Ultraviolet

U.V. detection was used early in normal phase ion-pairing partition studies.^{50,51} While measurements are usually made at 280 nm,^{52,53} 250 nm was used in a study in which norepinephrine enantiomers were separated using tetraacetylglucopyranosylisothiocyanate and triacetylpyranosylisothiocyanate derivitization.⁵⁴ Other studies were done optimizing the effect of the acid, the organic modifier and the pH of the mobile phase⁵⁵ and determining the reverse phase retention mechanism.⁵⁶

5.632 Fluorescence

A comprehensive review of fluorescence detection in catecholamine analysis by H.P.L.C. has recently been published.⁵⁷ Derivitization includes: trihydroxyindole,^{52,58,59,60,61} fluorescamine,^{62,63,64} and o-phthalaldehyde,^{58,65,66,67} pre and post-column. While THI detection was used in a direct injection continuous flow double ion-exchange column system,⁶⁸

TABLE IV

THIN LAYER AND PAPER CHROMATOGRAPHY OF LEVARTERENOL

<u>Medium</u>	<u>Solvent</u>	<u>Detection</u>	<u>Reference</u>
Avicel (microcrystal- line cellulose)	1st dimension: 1-butanol:MeOH: 1 N formic acid (60:20:20) 2nd dimension: CHCl ₃ :MeOH: 1N NaOH (60:35:5)	Diazotized p-nitroan- iline	43
Cellulose MN 300Ecteola (anion ex- changer)	1-butanol:EtOH: 0.5N HOAc (38: 8.5:20)	aqueous et- hylenediam- ine (1:4)	44
Cellulose phosphate paper	1-butanol:pyr- idine:H ₂ O (14:3:30) 1-butanol:EtOH (95%):H ₂ O (1:1: 1)	0.5% potas- sium ferri- cyanide 10% ferric chloride	45

TABLE V

GAS LIQUID CHROMATOGRAPHIC ANALYSIS of LEVARTERENOL

<u>Column</u>	<u>Detector</u>	<u>Derivitization</u>	<u>Detection Limits</u>	<u>Reference</u>
1% SE-30	electron capture	pentafluorobenzylimine - trimethylsilyl	100 pg	15
1% OV-17				
5% SE-30	electron capture	pentafluorobenzaldehyde	1.4×10^{-16} mol/sec	46
	flame ionization	bis-trimethylsilylacetamide	2.1×10^{-13} mol/sec	
3% OV-17	flame ionization	trimethylsilylimidazole	25 ng	47
7% DC-11	dual flame ionization	bis-trimethylsilylacetamide	0.1 pg	48
		trifluoroacetamide		
3% OV-1	electron capture	2,6-dinitro-4-trifluoromethylbenzenesulfonic acid	0.5 pg	49

TABLE VI

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SYSTEMS for LEVARTERENOL1. Ultraviolet Detection

<u>Column</u>	<u>Mobile Phase</u>	<u>Detection Limit</u>	<u>Reference</u>
Silica gel	BuOH:Hexane (1:1) EtOAc:Hexane (9:1) BuOH:MeCl ₂ (2:3) (0.1 M HClO ₄ stationary)	0.1 μ g	50
LiChrosorb SI 100	BuOH:MeCl ₂ (4:6) (0.25 M HClO ₄ stationary)	1.0 $\frac{\text{pmole}}{\text{ml}}$	51
μ Bondapak C ₁₈	0.17M HOAc pH 2.6	5 ng	52
Nucleosil C ₁₈	0.67M phosphoric acid	40 $\frac{\text{pg}}{\text{ml}}$	53
O.D.S.	10 mM phosphate buffer pH 2.8 : MeOH	-	54
Spherisorb 55 Silica Porasil- 60A Silica	aqueous perchloric, acetic, chloroacetic, dichloroacetic acids	-	55
LiChrosorp RP-8	0.1M H ₃ PO ₄ pH 3.0, Na octylsulfate: 1.15 % pentanol	-	56

TABLE VI (continued)

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SYSTEMS for LEVARTERENOL2. Fluorescence Detection

	<u>Column</u>	<u>Mobile Phase</u>	<u>Detection Limit</u>	<u>Reference</u>
	Zipax SCX	0.08M NaH ₂ PO ₄ pH 4.3	20 pg	58
	Zorbax ODS	0.1M NaH ₂ PO ₄ pH 3.15		
	Zipax SCX	0.15M NaH ₂ PO ₄	1 pg	59
	Zipax SCX	0.03M NaH ₂ PO ₄ :CH ₃ CN (60 g/L)	1 pg	60
572	Zipax SCX	50 mM NaH ₂ PO ₄ :CH ₃ CN (50 ml/L)	0.25 ng	61
	Hitachi 3011 gel 3010-OH	MeOH:Tris HCl pH 8 (7:3) MeOH:0.15M borate pH 8 (7:3)	0.1 nmol	62
	TSK gel 160	MeOH:Tris HCl 0.5M pH 8	100 ng	63
	TSK-LS 160	Tris HCl:CH ₃ CN (90:10) pH 8.4	50 μ g	64
	Zipax SCX	0.15M NaH ₂ PO ₄ pH 4.37	1 pg	65
	Bondapak SCX	Citric acid/HOAc/NaOAc/ NaOH pH 2.8	7.5 ng	67
	Zipax SCX- CDR-20 anion exchange	0.07M NaH ₂ PO ₄	10 pg	68

TABLE VI (continued)

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SYSTEMS for LEVARTERENOL2. Fluorescence Detection

<u>Column</u>	<u>Mobile Phase</u>	<u>Detection Limit</u>	<u>Reference</u>
TSK-LS 160	CH ₃ CN:0.05M imidazole HCl pH 7.3: Na ₂ EDTA 60 mg/L	25 fmol	69

3. Electrochemical Detection

Zipax SCX	0.1M HClO ₄	2 ng	74
Vydac SCX	0.01M H ₂ SO ₄ / 0.04 M NaSO ₄	0.5 ng/mL	75
Corasil/CX	acetate/citrate buffer pH 5.2	20 pg	76
C ₁₈	0.1M citric acid: 0.1M Na ₂ HPO ₄ (3:2) 0.3 mM Na octane sulfate	10 pg	77
Nucleosil SA	citrate/acetate buffer pH 5.2	80 fmol	78
Zipax SCX- Vydac SCX	citrate/acetate buffer pH 5.1	100 pg	79

TABLE VI (continued)

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SYSTEMS for LEVARTERENOL3. Electrochemical Detection

<u>Column</u>	<u>Mobile Phase</u>	<u>Detection Limit</u>	<u>Reference</u>
μ Bondapak C ₁₈	0.1M HNO ₃ pH 3.0 octane sulfate	-	80
μ Bondapak C ₁₈	citrate/phosphate buffer: MeOH (85:15) pH 3.3 Na octane sul- fonate 2.5×10^{-3} M EDTA	-	81
μ Bondapak C ₁₈	0.1MNaH ₂ PO ₄ 0.1M EDTA	100 fmol	82
Vydac SCX	citrate/acetate buffer	10 pg	83
μ Bondapak Phenyl	NaH ₂ PO ₄ buffer pH 5.5	20 pg	84
μ Bondapak C ₁₈	MeOH:H ₂ O pH 4.2 0.002 M PIC B7 or B8		
Whatman SCX	0.008M citric acid/ 0.013M acetate pH 5.2 0.01 mM EDTA	16 pg	85
Nucleosil SA	citrate/acetate buf- fer pH 5.2	0.25 nmol/L	86

TABLE VI (continued)

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SYSTEMS for LEVARTERENOL3. Electrochemical Detection

<u>Column</u>	<u>Mobile Phase</u>	<u>Detection Limit</u>	<u>Reference</u>
μ Bondapak C ₁₈ Ultrasphere ODS Spherisorb ODS Biophase ODS	0.1M phosphate buffer pH 2.8 Na octane sul- fate 9mg/L or 0.15M chloroacetic acid pH 3.1/1 mM EDTA/Na octane sulfate 25 mg/L	25 pg	87
μ Bondapak C ₁₈	0.1M citrate:0.1M phosphate buffer (300: 160) Na octane sulfate 0.01 g/L	1 μ g/L	88
μ Bondapak C ₁₈	NaOAc pH 4.8/ 7% CH ₃ CN, Na heptane sulfonate	50 pg	94
Ultrasphere ODS	citrate/phosphate buf- fer pH 4.85/ 14% MeOH 3 mM Na octane sulfate	250 pg/mL	95

TABLE VI (continued)

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SYSTEMS for LEVARTERENOL3. Electrochemical Detection

<u>Column</u>	<u>Mobile Phase</u>	<u>Detection Limit</u>	<u>Reference</u>
Zipax SCX	0.1M HClO ₄	0.03 pmol	96
Yanapak ODS-Al ₂ O ₃	Britton-Robinson buffer pH 1.8/0.5 mM Na heptanesulfon- ate and Tris pH 8.8/0.25% EDTA	40 ng	97
Nucleosil C ₁₈	citrate/phosphate buf- fer pH 6.5/MeOH	500 pg	98
Nucleosil C ₁₈	MeOH: citrate/phosphate buffer pH 5.2 (1:20)	1 ng/mL	99

a chemiluminescent fluorescence detector has been utilized by reacting bis(2,4,6-trichlorophenyl) oxalate with fluorecamine labeled levarterenol. This method gave a 20-fold increase in sensitivity over conventional fluorecamine labeling.⁶⁹

5.633 Electrochemical

The field of electrochemical detection of oxidizable compounds separated by H.P.L.C. has seen a rapid expansion in recent years following early work of P.T. Kissinger et al. Recent reviews of electrochemical detection of catecholamines include the following:⁷⁰⁻⁷³. The electrochemical detector in general consists of a thin-layer or wall-jet section connected to a reservoir containing the reference (Ag/AgCl or S.C.E.) electrode plus an auxiliary electrode. The working electrode or anode placed in the thin-layer (0.002-0.015 in.) section consists of a well filled with carbon paste, a glassy carbon electrode or a gold/mercury electrode. A number of studies have been conducted using each of these electrodes for the detection of levarterenol. References using carbon paste electrodes include: ⁷⁴⁻⁹⁰, while glassy carbon electrodes were used in ⁹¹⁻⁹⁵. In addition dual carbon paste ⁹⁶ and dual glassy carbon electrodes ⁹⁷ have been used. A recent development is the use of a rotating disc electrode with which it is possible to decrease the diffusion layer thickness, thus increasing the effective detection volume and sensitivity.^{98, 99}

5.634 Radioenzymatic

The products of the radio-enzymated reaction of levarterenol in the system described in 5.4 above using catechol-O-methyltransferase have been separated by H.P.L.C. with normal phase ⁵¹ and reversed-phase systems. ^{53,100} Following fraction collection, samples were counted by liquid scintillation.

6. Metabolism and Pharmacokinetics

The metabolism of levarterenol has been outlined in the original monograph. ⁵ Little was known of the pharmacokinetics of this drug however until the very sensitive methods presently available were developed. One difficulty encount-

ered in these measurements is a distinction which must be made between endogenous norepinephrine and its metabolites and the exogenous drug. This is normally done by radioactive labeling. Early human studies were done with the administration of ^3H -d,l-norepinephrine and the collection of urinary excretion data. A triexponential expression with a three compartment model explained the data when $0.02\text{ }\mu\text{g/kg}$ was infused in one hour. The half-lives obtained from the three sections of the urinary specific activity/time curve were: 1.19 hr., 5.22 hr., and 23.43 hr. The rate of endogenous norepinephrine output was found to be $0.022\text{ }\mu\text{g/mg creatinine}$.¹⁰¹ A six compartment model with a biexponential equation was used to explain other urinary excretion data.¹⁰² Here $8.3 \times 10^{-6}\text{ }\mu\text{g/kg min}$ d,l-norepinephrine was administered for 48 hours. An average of $27\text{ }\mu\text{g}$ of free Nor-epi appeared in the urine with $5814\text{ }\mu\text{g}$ of metabolites in 24 hours. In these two studies fluorescence and liquid scintillation measurements were used.

More recently ^3H -levarterenol itself has been administered by infusion and plasma kinetic data obtained using the sensitive radioenzymatic methods. Results obtained are shown in Table VII. Levarterenol levels have been determined along with metabolites in human urine, plasma and CSF using several of the methods discussed above. These are summarized in Table VIII.

7. Acknowledgement

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TABLE VII

LEVARTERENOL PHARMACOKINETICSZERO-ORDER INFUSION PLASMA DATA

<u>Radio-enzyme Method</u>	<u>Infusion Rate</u>	<u>Infusion Duration</u>	<u>Average $t_{1/2}$ min</u>	<u>Total Clearance L/min</u>	<u>Endogenous Production Rate</u>	<u>Average Plasma Level pg/mL</u>	<u>Reference</u>
COMT	0.002 μ g/min	90 min	-	2.8	0.54 μ g/ m ² min	240	103
PNMT	0.01-0.075	10 min	1.45	4.5	0.83-0.85	190	104
	0.06 μ g/ kg min	10 hr	2.09		μ g/min		
COMT	0.1 - 5 μ g/min	60 min	2.4	3.07	0.7 μ g/ min	228	105

TABLE VIII

REFERENCES for LEVARTERENOL ANALYSIS in HUMAN FLUIDS

<u>Method</u>	<u>Urine</u>	<u>Plasma</u>	<u>CSF</u>
fluorescence	33	33	
GLC	48	48	
radio-enzym- atic		36, 40, 41	40
HPLC	52, 61, 64, 69 90, 97, 110	53, 60, 85, 86, 87, 93, 94, 95, 100	

REFERENCES for LEVARTERENOL METABOLITE ANALYSIS in HUMAN FLUIDS

<u>Method</u>	<u>Urine</u>	<u>Plasma</u>	<u>CSF</u>
GC-MS	106, 107, 108	107	
UV	109		
HPLC			111

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The present literature review includes material published through December, 1981.

MEPROBAMATE

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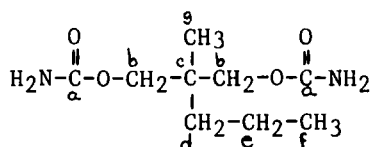
The following supplement contains updated information pertaining to the analytical chemistry of meproba-mate. A literature survey was conducted and is complete up to January 1981. The numbering system for topics discussed is the same as that in the original profile (Volume 1, pp. 207-232).

2. Physical Properties

2.2 Nuclear Magnetic Resonance Spectra

The carbon-13 NMR spectrum of a Wyeth In-House Reference Standard in deuterated dimethyl sulfoxide as obtained on a Varian FT-80-A spectrometer is presented in Figure 1. The assignment of the individual signals is given in the following table⁽¹⁾.

<u>Carbon</u>	<u>Chemical Shift (ppm)</u>
a	158.39
b	68.31
c	37.73
d	36.84
e	16.42
f	15.17
g	18.99



2.7 Crystal Properties

2.71 Polymorphism

Three crystal modifications were investigated by Burger and Schulte (2) and two modifications were investigated by Clements and Popli (3). Both references discussed thermal properties, infrared spectra, X-ray diffraction patterns and dissolution studies.

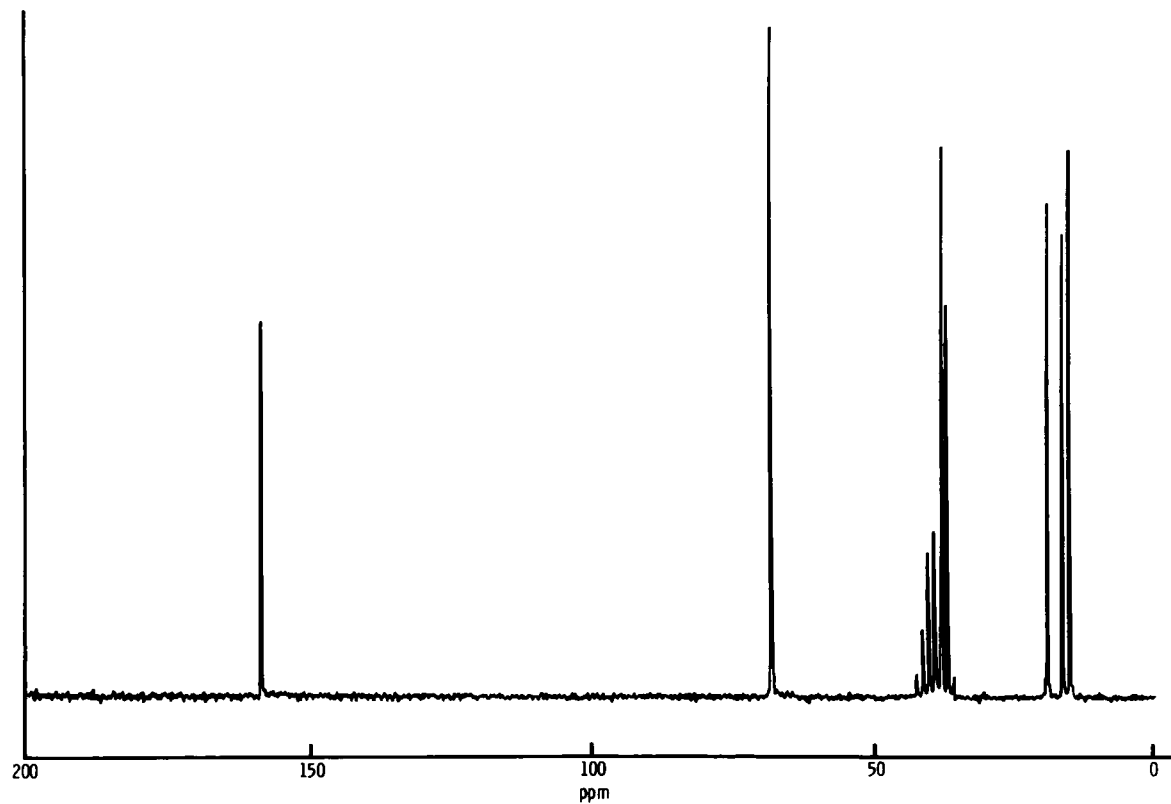


Figure 1 - ^{13}C NMR Spectrum of Meprobamate

6. Methods of Analysis

6.5 Titrimetric

Meprobamate, upon hydrolysis in acidic solution forms in a stoichiometric amount ammonia, which can be determined, after making the solution alkaline, by an ammonia sensing electrode⁽⁴⁾. Likewise when meprobamate is hydrolyzed in a basic solution carbon dioxide is formed, which can be determined by a carbon dioxide sensing electrode⁽⁵⁾.

6.64 Column chromatography

Meprobamate has been determined by HPLC⁽⁶⁾ using as the eluant varying proportions of chloroform mixed with carbon tetrachloride, hexane or butyl ether and a column consisting of small particle fully porous silica packing material or a monomolecular layer of a cyanopropylsilane chemically bonded to a small particle, fully porous silica support. The eluted peaks were monitored using differential refractive index detection.

Meprobamate has also been assayed by HPLC after alkaline hydrolyses, to 2-methyl-2-propyl-1,3-propandiol and preparing the benzoyl ester of the diol⁽⁷⁾. An eluant consisting of 60 parts acetonitrile, 30 parts methanol and 30 parts water eluted the benzoyl derivative from a 15 cm x 4.6 mm Ultrasphere RP 18 column in 6 minutes at a flow rate of 2.5 ml/min. This procedure was used for analyses of meprobamate in plasma.

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TRIAMCINOLONE

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The following supplement contains updated information pertaining to the analytical chemistry of triamcinolone. A literature survey was conducted and is complete up to July 1981. The numbering system for topics discussed is the same as that in the original profile (Volume 1, pp. 367-396).

1. Description. Triamcinolone is a glucocorticoid used primarily in the treatment of adrenocortical and rheumatic disorders.

1.13 The CA registry number for triamcinolone is 124-94-7.

2. Physical Properties

2.1 Infrared Spectra

The original profile¹ contained a comparison of infrared spectral assignments of triamcinolone in mineral oil with that of the compound in the solid state. Bellomonte² published the infrared spectra (KBr disc) of 13 fluorinated steroids, including triamcinolone and triamcinolone acetonide, and discussed the special influence of the fluorine atom. The major assignments, which are in excellent agreement with Florey¹, are supplemented with the assignments of the C-F stretching frequency; i.e., 1065 (1⁰) and 979 (2⁰) cm⁻¹ for triamcinolone and 1080 (1⁰) and 972 (2⁰) cm⁻¹ for triamcinolone acetonide.

2.2 Nuclear Magnetic Resonance

The improved ¹H-NMR and the ¹³C-NMR spectra³ of triamcinolone are shown in Figures 1 and 2, respectively (please refer to Figure 4, Section 2.2 of the original profile¹ for comparison and assignments of the ¹H-NMR spectrum). The ¹³C-spectrum was determined on a JEOL FX60Q spectrometer using a 10mm C/H dual probe. A complete assignment for all the carbon atoms in the ¹³C-NMR spectrum is listed in Table I. The assignments are based on relative chemical shifts (dimethylsulfoxide-d₆ = 39.5 ppm) and ¹³C-¹⁹F coupling constants and are consistent with literature values for closely related compounds.⁴ The assignments were simplified by the utilization of polarization transfer to selectively enhance and phase alter individual carbon types.⁵

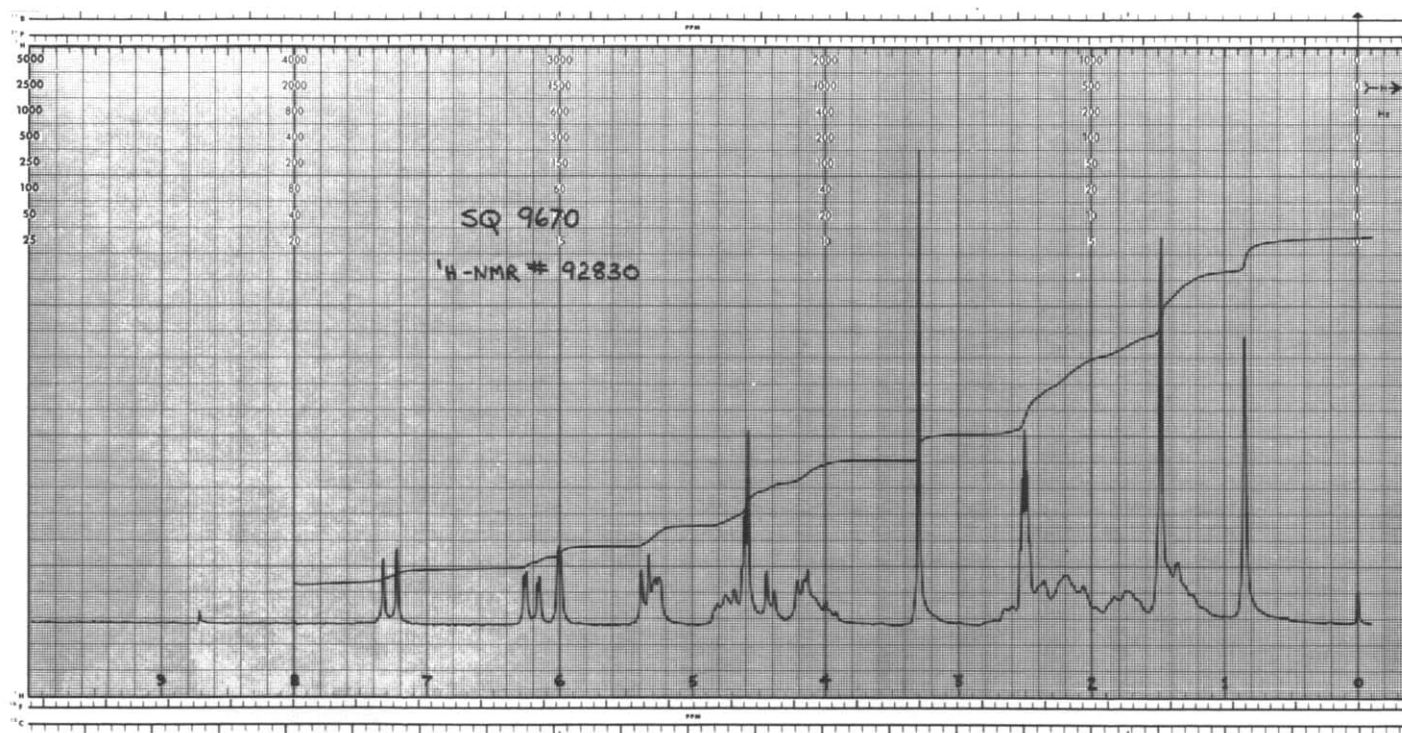


Figure 1. ^1H -Nuclear Magnetic Resonance Spectrum of Triamcinolone (SQ 9670) in Dimethylsulfoxide- d_6 . Instrument: Varian XL-100A.

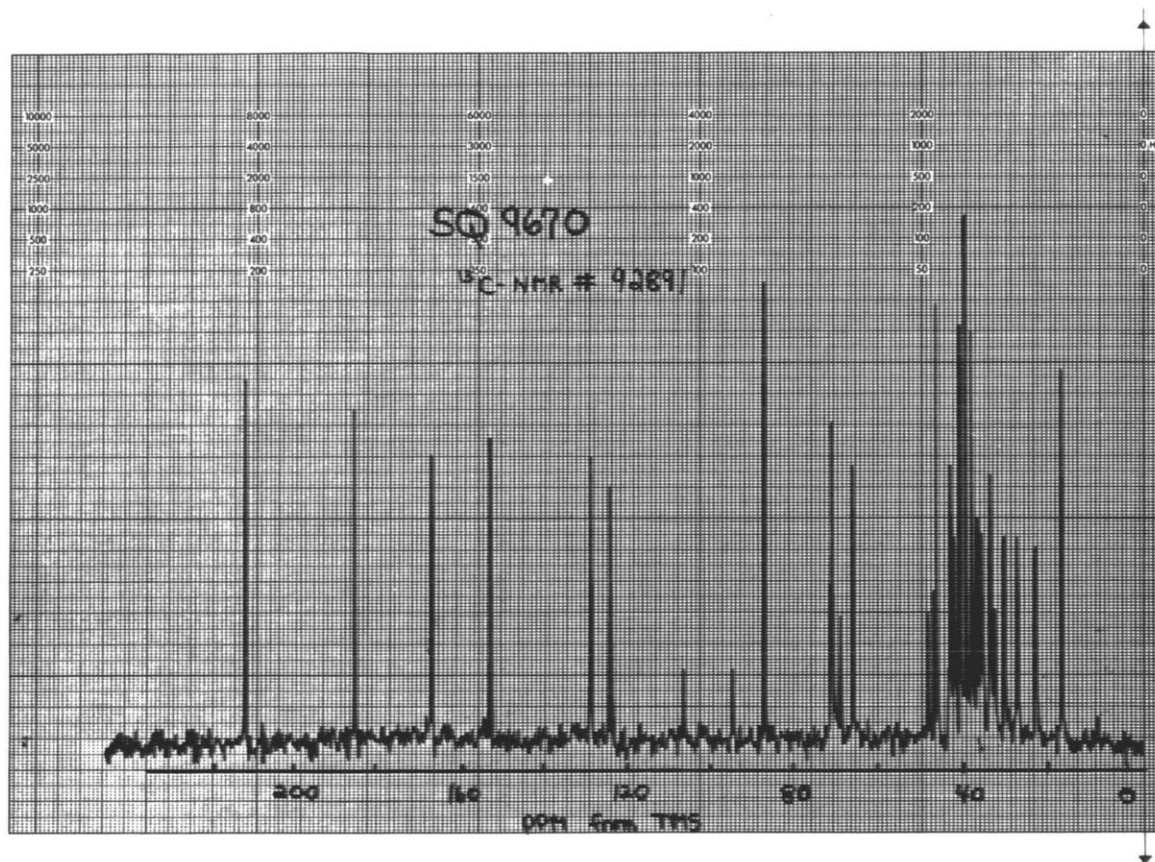


Figure 2. ^{13}C -Nuclear Magnetic Resonance Spectrum of Triamcinolone (SQ 9670) in Dimethylsulfoxide. Instrument: JEOL FX60Q.

TABLE I

¹³C-NMR Spectral Assignments^a of Triamcinolone

<u>Carbon Number</u>	<u>Chemical Shift^b</u>	<u>Carbon Number</u>	<u>Chemical Shift</u>
1	152.7	12	35.9
2	129.1	13	46.2
3	185.4	14	43.2
4	124.2	15	33.6
5	167.0	16	71.4
6	30.3	17	87.6
7	27.3	18	16.7
8	33.3(19.6) ^c	19	22.1(5.8)
9	101.1(174.8)	20	211.6
10	48.0(22.4)	21	66.6
11	70.6(37.1)		

^aAll chemical shifts are in ppm from tetramethylsilane (TMS) with internal reference dimethylsulfoxide = 39.5 ppm. ^bAll spectra were run in DMSO-d₆. ^c¹³C-¹⁹F coupling constants shown in parentheses.

2.4 Mass Spectra

The low resolution mass spectra of 28 corticosteroid 21-esters and related compounds of pharmaceutical interest have been determined by Toft and co-workers⁶ on an AEI MS-12 via direct probe. The mass spectra of triamcinolone and triamcinolone diacetate are in excellent agreement with those published in the original profile.

2.9 Solubility Data

2.91 Solubility

Solubilization of 19 steroid hormones, including triamcinolone, triamcinolone acetonide and triamcinolone diacetate by polyoxyethylene lauryl ether was reported by Tomida⁷ who also concluded that the solubilization of steroids by polyoxyethylene lauryl ether micelles is directly dependent upon their lipophilicity. Triamcinolone was found to have an aqueous solubility of 2.07×10^{-4} M.

2.92 Partition Coefficients

The role of crystal structure (as reflected by the melting point and the entropy of fusion)

and of the activity coefficient (as reflected by the octanol-water partition coefficient) in controlling the aqueous solubility of either liquid or crystalline organic nonelectrolytes, including triamcinolone, triamcinolone acetonide and triamcinolone diacetate was discussed by Yalkowsky and Valvani.¹⁰ Structural relationships between a large group of steroids, including triamcinolone and triamcinolone acetonide, and their ether-water partition coefficients were explored by Flynn.¹¹ Correlation with biological activity was also discussed.

The partition coefficients of triamcinolone in several systems have been reported by Tomida.⁷ He determined that the aqueous-micellar, octanol-water and ether-water partition coefficients were 96.3, 10.8 and 0.757, respectively. These values have been corroborated by several other investigators.⁸⁻¹¹

2.10 Crystal Properties

The mean diamagnetic susceptibility of 20 corticosteroids, including triamcinolone, was determined by the Faraday method.^{12,13} The orientation of the principal molecular axes with regard to the steroid skeleton was also given.

3. Synthesis

Barton and Heese¹⁴ developed and patented a procedure for the chemical conversion of Δ^1 -dehydrotriamcinolone to triamcinolone using triphenylmethyllithium in combination with lithium aluminum hydride or $\text{LiH}_2\text{Al}(\text{OCH}_2\text{CH}_2\text{OCH}_3)_2$ at -78°C .

Numerous microbiological dehydrogenations have been published or patented on the conversion of Δ^1 -dehydrotriamcinolone to triamcinolone.¹ Maximization of this conversion was reported by Yoshida and co-workers¹⁵ using a mixed culture system. Modification and further improvement of the dehydrogenation was published¹⁶ and patented¹⁷ by Ryu and co-workers using a semicontinuous enzymatic process where the concentration of enzyme, active 3-ketosteroid Δ^1 -dehydrogenase, is increased in parallel with that of the steroid. Any water-soluble 16,17-cycloborate of a steroid of the $16\alpha,17\alpha$ -dihydroxy-3-keto- Δ^4 -pregnene series will work in this system.

5. Drug Metabolism and Pharmacokinetics

5.1 Drug Metabolism

Yamashita¹⁸ studied the metabolism of seven synthetic corticoids, including triamcinolone in rat liver. He found that the metabolism of the corticoids by rat liver slices or homogenates proceeded at rates inversely proportional to the anti-inflammatory potency of the compounds. The major metabolic pathway for triamcinolone was found to be 6 β -hydroxylation. In addition, the liver material also metabolized triamcinolone to the 20-dihydro and the 11-dihydro derivatives.

5.2 Pharmacokinetics and Bioavailability

The permeability of the cornea to topically applied drugs, including triamcinolone, has been extensively reviewed by Benson.¹⁹ Triamcinolone was usually determined by the blue tetrazolium assay (section 6.31). Local changes in the skin and subcutaneous tissues are known to occur as a result of percutaneous absorption of corticosteroids. This was the subject of an extensive review article by Keipert²⁰ and included discussions of triamcinolone and triamcinolone acetate. The vasoconstrictor assay was the assay method of choice in these studies.

The interaction between different corticosteroids, including triamcinolone, and commonly administered antacids and its implication on bioavailability was studied by Naggar, et al.²¹ Triamcinolone was found to be quantitatively adsorbed on charcoal and magnesium trisilicate and not adsorbed at all on magnesium or calcium carbonates.

The role of bioavailability of steroids on their lipophilic character is of extreme importance. Biagi and co-workers²² showed that the chromatographic R_m value of triamcinolone and several other steroids could be correlated with its lipophilic character. One important conclusion arising from this study is that the dependence of protein binding absorption and biotransformation on lipophilic character might strongly influence the availability of steroids at the site of action.

The effects of fourteen steroids, includ-

ing triamcinolone, on the NMR spectra of liposomes derived from egg yolk phosphatidyl cholines were studied by Ahmad and Mellors²³ using continuous wave and Fourier-transform measurements at 60 MHz. The steroids were compared for their ability to broaden the acyl methylene resonances of phosphatidylcholine, when incorporated into liposomes at 25% molar ratio. This study confirmed the importance of the steroid side-chain at C-17 as a requirement for sterol-phospholipid interaction.

6. Methods of Analysis

6.3 Colorimetric Analysis

6.31 Occhipinti and co-workers²⁴ reported significant improvements in the tetrazolium blue method for the determination of steroids containing an α -ketol side chain. They reported optimal concentration and experimental conditions for rapid quantitative determinations of seventeen representative steroids including triamcinolone and triamcinolone acetonide, occurring in pharmaceutical formulations. Tsuchikura and co-workers²⁵ found that the 1,2-double bond, 16 α -hydroxy and 16,17-acetonide groups inhibited the blue tetrazolium reactions of steroids.

The stoichiometry and mechanism of the reaction of blue tetrazolium with triamcinolone was studied by Görög and Horvath.²⁶ On the basis of previous literature data^{27,28} and experimental results, they concluded that, initially, the side chain was oxidized to the 20-keto-21-aldehyde. This oxidation was followed by the base-catalyzed D-homo rearrangement and subsequent oxidation by blue tetrazolium to form an α -diketone (D ring), which existed in the enolic form.

6.32 Tsuchikura and co-workers²⁵ found that the 1,2-double bond, 16 α -hydroxy and 16,17-acetonide groups inhibited the isonicotinic acid hydrazide reaction of steroids. Yamashita and co-workers²⁹ investigated the qualitative and quantitative determination of synthetic corticoids and reported the isonicotinic acid hydrazide determination of triamcinolone and triamcinolone acetonide.

6.33 The colorimetric determination of triamcinolone using its reaction with dinitro-

phenylhydrazine has been reported.²⁹

6.34 The reaction of a 1 mg/ml aqueous solution of sulfuric acid with triamcinolone produced a yellow color in daylight and no color when viewed with an ultraviolet lamp at 366 nm.³⁰ Triamcinolone did not give a color reaction when treated with vanillin and sulfuric acid.³¹

6.35 The characterization of glucocorticoids, including triamcinolone, with Dische's reagent (diphenylamine in acetic acid and sulfuric acid) or with a modified Dische's reagent (N-methyl-diphenylamine in acetic acid and sulfuric acid) was described by Rioux-Lacoste and Viel.³² The steroids were dissolved in 20% acetic acid solutions, reacted with the reagent at 85° C. and the absorbance determined spectrophotometrically between 600-650 nm.

6.36 The presence of substituents at C-16 greatly reduces the chromogenicity of 17 α -corticosteroids in the Porter-Silber test (phenylhydrazine and sulfuric acid).¹ Although this test has been used for the determination of triamcinolone,^{25,29} it is not recommended because of the low molar absorptivity ($E_{1\text{cm}}^{1\%} = 37$ @ 420 nm)³⁰ of the chromogen produced.

6.37 Triamcinolone has been quantitated by its reaction with 2,3,5-triphenyltetrazolium chloride followed by absorbance measurement.³³ Triamcinolone was also determined spectrophotometrically with 2,3,5-triphenyltetrazolium chloride by Smockkiewicz and Jasiczak.³⁴ They found that the absorption of light at 490 nm was much higher for compounds with neighboring OH groups; i.e., 16 α -17 α -diol, than for steroids without an OH group at C-16. A 5% solution of 2,3,5-triphenyltetrazolium chloride in methanol has been used as a spray reagent for the detection of triamcinolone in thin layer chromatographic systems.³⁴

6.38 A process method has been developed by Ivashkiv³⁵ for rapidly estimating the conversion of 9 α -fluoro-16 α -hydroxyhydrocortisone to triamcinolone in fermentation broths. The conversion of the steroid was estimated by measuring the absorb-

ance of the aqueous steroid borate complex at 241.5 and 271 nm and calculating the ratio.

6.4 Polarographic Analysis

Differential pulse polarography was used to study the electroanalytical behavior of triamcinolone.³⁷ The half wave potentials vs. the Ag/AgCl electrode of triamcinolone were determined as -1.50V (C-3 keto reduction) and -1.70V (C-3 keto reduction) in Britton-Robinson buffer pH 10 (50% v/v in methanol), -1.56V (C-3), -1.75V (C-3) and -1.95 (C-20 keto reduction) in 0.03M tetramethylammonium hydroxide in methanol and -1.70V (C-3), -1.98V (C-3) and -2.20V (C-20) in 0.02M tetramethylammonium hydroxide in aqueous DMF (87% v/v). This electroanalytical technique was then used to determine triamcinolone in single-component tablets³⁸ and later in multicomponent and complex pharmaceutical preparations.³⁹

6.5 Chromatographic Analysis

6.52 Thin layer chromatographic analysis

A compilation of the chromatographic identification and separation of triamcinolone and other corticosteroids of similar structure is shown in Table II. This data has been published after 1970.

6.53 Column Chromatography

The retention characteristics of 53 steroids on an acetonitrile-diatomaceous earth column are given for elution with n-heptane followed by stripping with chloroform.⁵³ Triamcinolone was recovered nearly quantitatively (98.4%) in the chloroform.

6.54 High Performance Liquid Chromatography

A compilation of the high performance liquid chromatographic separation systems for triamcinolone published after 1970 is shown in Table III.

6.55 Gas-Liquid Chromatography

Several gas liquid chromatographic systems for the separation and identification of triamcinolone with and without prior derivatization are shown in Table IV. In addition, a gas chromato-

TABLE II

Thin Layer Chromatographic Systems for the Detection and Determination of Triamcinolone

<u>Solvent System</u>	<u>Adsorbent</u>	<u>Detection</u>	<u>R_f</u>	<u>Reference</u>
Methylene chloride:diethyl ether:methanol:water (77:15:8:1.2)	Kieselgel GF254	UV at 254 nm	0.15	47
1,-1-Dichloroethanol:acetone:acetic acid (160:40:10)			0.16	
Chloroform Stationary phase-Formamide:acetone (10:90)			0.03	
Methylene chloride:p-dioxane:water (10:5:5)-lower layer	Silica gel	Spray with 2,3,5-triphenyl-2H-tetrazolium chloride in methanol, heat at 110° C. for 10 minutes. Red-pink color	0.30	36
Chloroform saturated with ammonia-methanol (18:1)	Kieselgel GF254	Spray with 50% ethanolic sulfuric acid spray, heat at 120° C. for 5 minutes, UV quench or iodine chamber	0.06	48
Acetone:12N ammonia (99:1)			0.75	

TABLE II (continued)

Thin Layer Chromatographic Systems for the Detection and Determination of Triamcinolone

<u>Solvent System</u>	<u>Adsorbent</u>	<u>Detection</u>	<u>R_f</u>	<u>Reference</u>
Ethanol:5N ammonia (9:1)			0.58	
Methylene chloride:dioxane: water (10:5:5)-lower layer			0.40	
Ethylene chloride:methanol: water (95:5:0.2)			0.05	
Methylene chloride:dioxane: water (10:5:5)-lower layer	Silica gel 60	UV at 254 nm	0.22	49
Methylene chloride:dioxane: water (12:3:5)-lower layer			0.07	
Butyl acetate or Dichloromethane:methyl acetate: water (2:1:1)-lower layer Stationary phase - Dioxane	Kieselgel 60 F254	Spray with 20% anhydrous zinc chloride in methanol; heat at 110° C. for 20 minutes. UV at 254 and 366 nm	Identity Test	50
Chloroform:methanol:glacial acetic acid (90:10:2)	Silica gel	UV at 254 nm	0.20	51

TABLE II (continued)

Thin Layer Chromatographic Systems for the Detection and Determination of Triamcinolone

<u>Solvent System</u>	<u>Adsorbent</u>	<u>Detection</u>	<u>R_f</u>	<u>Reference</u>
Chloroform Stationary phase - Formamide:acetone (90:10)	Kieselguhr G	Spray with sulfuric acid (1 mg/ml) UV at 366 - violet color	0.24	30
Cyclohexane:ethyl acetate: water (25:75:1)			0.15 - 0.21	
Methylene chloride:diethyl- ether:water (77:15:8:1.2)			0.04	
Chloroform:methanol: acetic acid (90:10:2)	Silica gel	Fluorescence at 2537 Å ⁰	0.20	52

TABLE III

High Performance Liquid Chromatographic Separation Systems for Triamcinolone

<u>Column</u>	<u>Mobile Phase</u>	<u>Flow Rate (ml/min)</u>	<u>Retention Time (min) or relative</u>	<u>Detection (nm)</u>	<u>Ref.</u>
Diol (polar coated silica-5 μ) 250 x 4.5mm ID	n-hexane:isopropanol (75:25)	1.3	20.0	254	54
μ Bondapak C ₁₈ 10 μ 30cm x 3.9mm ID	3.3g potassium phosphate dibasic 4.2g potassium phosphate monobasic 2.8L methanol 1.2L water	2.0	1.74	254/280	55
10 μ Silica gel 25cm x 0.2cm ID	95% ethanol:methylene chloride (5:95)	1.55	4.90	254	56
Spherisorb S10-ODS	methanol:water (56:44)	1.3 to 1.5	4.5	254	57
Corasil II 2 ft. x 2.3mm ID	n-hexane:ethyl acetate (3:2)	1.0	9.05	254	58
Bondapak C ₁₈ / Corasil 2 ft. x 2.3mm ID	methanol:water (2:3) acetonitrile:water (1:9) acetonitrile:water (1:4)	1.5	0.4 1.0 0.4		

TABLE III (continued)

High Performance Liquid Chromatographic Separation Systems for Triamcinolone

Column	Mobile Phase	Flow Rate (ml/min)	Retention Time (min) or relative	Detection (nm)	Ref.
μ Bondapak C ₁₈ 30cm x 4.0mm ID	methanol:water (70:30)	1.5	1.14	254	59
	methanol:water (50:50)		1.86		
	acetonitrile:water (60:40)		1.00		
	acetonitrile:water (40:60)		1.19		
			relative to acetone 1.00		
Bondpak C ₁₈ / Corasil 61cm x 2.3mm ID	methanol:water (60:40)	1.5	1.06		
	methanol:water (40:60)		1.76		
	acetonitrile:water (40:60)		0.94		
	acetonitrile:water (20:80)		1.59		
			relative to acetone 1.00		
Bondapak Phenyl/ Corasil 61cm x 2.3mm ID	methanol:water (60:40)	1.0	0.98	254	
	methanol:water (40:60)		1.56		
	acetonitrile:water (40:60)		1.04		
	acetonitrile:water (20:80)		1.54		
			relative to acetone 1.00		
Zorbax SIL 3.8mm ID	cyclohexane:methylene chloride:ethanol (9:4:1)	50 kg/cm ²	28	254	60

TABLE IV
Gas Liquid Chromatographic Systems
for the Separation and Identification of Triamcinolone

<u>Column</u>	<u>Column T</u> <u>(°C)</u>	<u>Detection</u> <u>Carrier Gas,</u> <u>Flow Rate</u>	<u>Derivatization</u>	<u>R_f</u>	<u>Ref.</u>
2.5% SE 30 on 80/100 Chromosorb G 2 ft. x 4mm	250	FID N ₂ , 40 cc/min	None	1.13 Relative to cholesterol 1.00	61
1% OV-17 on 100/120 silanized Supasorb 9 ft. x 0.25 in.	260	EC N ₂ , 100 cc/min	trimethyl- silyl	23.6 min.	62
3% OV-17 on Chromosorb WHP 1.8m x 2.0mm	260	FID He, 60 cc/min	trimethyl- silyl	3.53 min	63

graphic analysis of steroids, including triamcinolone, in pharmaceutical products has been reported by Garzo and co-workers.⁶⁴

6.6 Fluorimetric Analysis

Kadin⁴⁰ has described a fluorimetric determination of several corticosteroids, including triamcinolone and triamcinolone acetonide, containing the $\Delta^{1,4}$ -3-keto group. These A ring dienone-containing steroids were reacted with zinc dust and 40% sulfuric acid in n-butyl ether. The fluorescence emission and activation maxima were then determined at 390 and 340 nm, respectively.

6.7 Titrimetric Analysis

Triamcinolone has been determined by combustion followed by titration of the resulting fluoride ion with thorium nitrate solution and alizarin red S indicator.⁴¹

Steroids with a 16α , 17α -diol group can be titrated with lead tetraacetate in acetic acid to a potentiometric endpoint. The micro version of this method using a 0.001N lead tetraacetate solution enabled triamcinolone contaminant to be determined in its $16,17$ -acetonide.^{42,43}

6.8 Differential Borohydride Analysis

The differential borohydride assay developed by Görög⁴⁴ is an excellent method for the determination of Δ^4 - and $\Delta^{1,4}$ -3-ketosteroids in pharmaceutical preparations. Addition of propylene glycol to the reaction mixture to complex sodium metaborate allowed Kirschbaum⁴⁶ to improve the reduction of Δ^4 - and $\Delta^{4,6}$ -3-ketosteroids. However, $\Delta^{1,4}$ -3-ketosteroids, i.e. triamcinolone, were less than 10% reduced with sodium borohydride. By the use of lithium borohydride instead of sodium borohydride, Chafetz and co-workers⁴⁵ were able to determine triamcinolone.

6.9 Radioimmunoassay

A radioimmunoassay for the determination of picogram quantities of triamcinolone in plasma was reported by Loo and Jordan.⁶⁵ The antibody against triamcinolone was obtained by immunizing rabbits with triamcinolone 21-hemisuccinate coupled to bovine serum albumin. The problem of antiserum cross reactivity (5%) with hydrocortisone was

TABLE V

Determination of Triamcinolone in Biological Fluids and Tissues

<u>Chromatographic Method</u>	<u>Extraction</u>	<u>Biological System</u>	<u>Internal Standard</u>	<u>Ref.</u>
GLC with derivatization	Ethyl acetate followed by partitioning between hexane and 70% methanol	Rat muscle	³ H-triamcinolone	62
GLC with derivatization	Ethyl acetate	Culture media of mouse and human dermal fibroblasts	Progesterone	63
GLC	Chloroform	Blood, urine and body tissues	Cholesterol	61
HPLC	Diethylether	Human serum	Prednisone	54
TLC with fluorescence	Dichloromethane	Plasma and urine	³ H-triamcinolone	51,52

circumvented by the transformation of the steroids to their Girard T hydrazones in ethanol denatured plasma. Triamcinolone reacted very slowly with Girard T.

7. Determination in Body Fluids and Tissues

Triamcinolone has been determined in biological fluids and tissues by several different methods of analysis (see Section 6.9) in combination with various extraction techniques. Table V summarizes the results that have been published since 1970. In addition, Saito and co-workers⁶⁰ have described a high pressure liquid chromatographic method for the separation and quantitation of 12 synthetic corticosteroids in blood and urine. Triamcinolone administration produced serum peaks at 2-5 hours with $t_{1/2} = 4$ hours.

8. Acknowledgements

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TRIAMCINOLONE ACETONIDE

David H. Sieh

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The following supplement contains updated information pertaining to the analytical chemistry of triamcinolone acetonide. A literature survey was conducted and is complete up to July 1981. The numbering system for topics discussed is the same as that in the original profile (Volume 1, pp. 397-422).

1. Description

Triamcinolone acetonide is a glucocorticoid used mainly in the treatment of adrenocortical and rheumatic disorders.

1.13 The CA Registry Number for triamcinolone acetonide is 76-25-5.

2. Physical Properties

2.1 Infrared Spectra

The original profile¹ contains a comparison of infrared spectral assignments of triamcinolone acetonide in mineral oil with that of the compound in the solid state. Bellomonte² has published the infrared spectra (potassium bromide disc) of 13 fluorinated steroids, including triamcinolone and triamcinolone acetonide, and discussed the special influence of the fluorine atom. The major assignments, which are in excellent agreement with Florey¹, are supplemented with the assignments of the C-F stretching frequency i.e., 1080 (1°) and 972 (2°) cm⁻¹ for triamcinolone acetonide.

Triamcinolone acetonide has been identified by a combination of optical rotation and infrared spectroscopy³.

2.2 Nuclear Magnetic Resonance Spectra

The improved ¹H-NMR and the ¹³C-NMR spectra⁴ of triamcinolone acetonide are shown in Figures 1 and 2, respectively (refer to Figure 2, Section 2.2 of the original profile¹ for comparison and assignments of the ¹H-NMR spectrum). The ¹³C-spectrum was determined on a JEOL FX60Q spectrometer using a 10 mm C/H dual probe. A complete assignment for all the carbon atoms in the ¹³C-NMR spectrum is listed in Table I. The assignments are based on relative chemical shifts

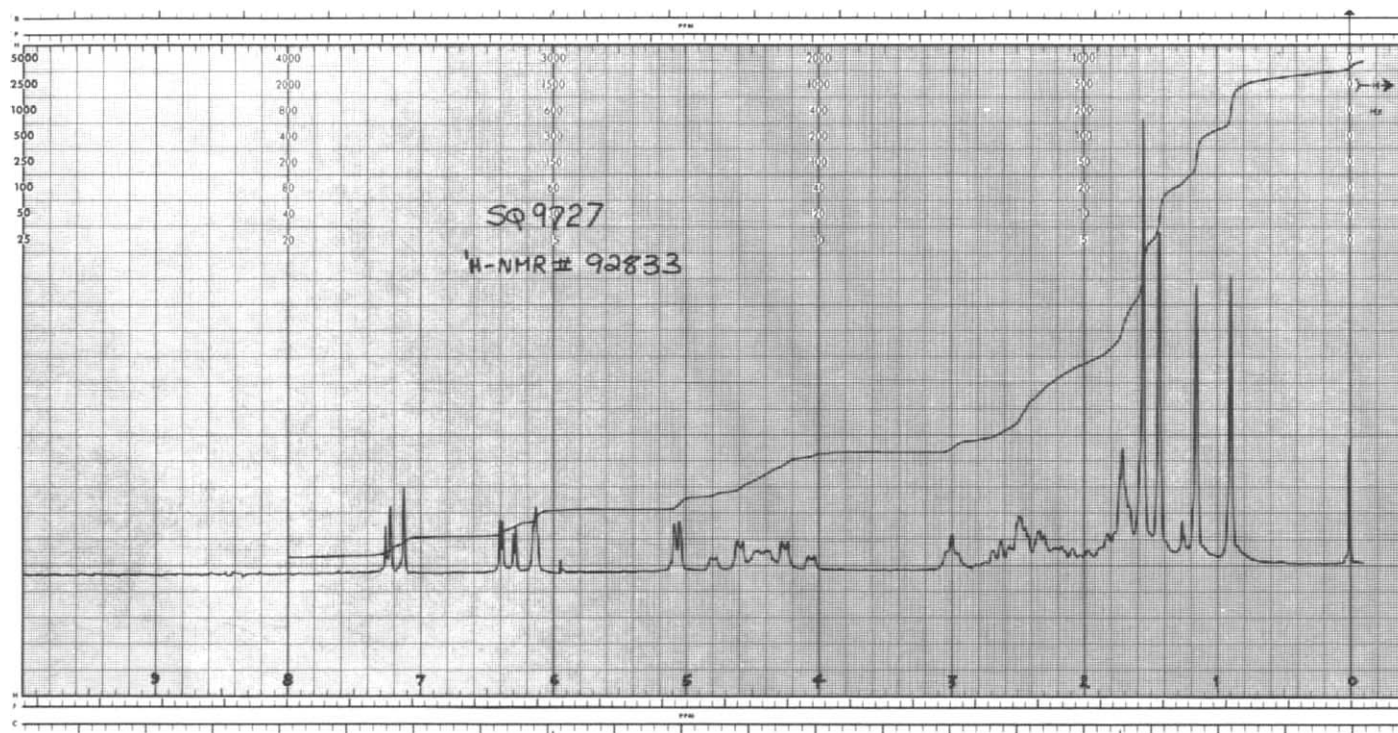


Figure 1. ^1H -Nuclear magnetic resonance spectrum of triamcinolone acetonide (SQ 9727) in CDCl_3 . (Instrument: Varian XL-100A).

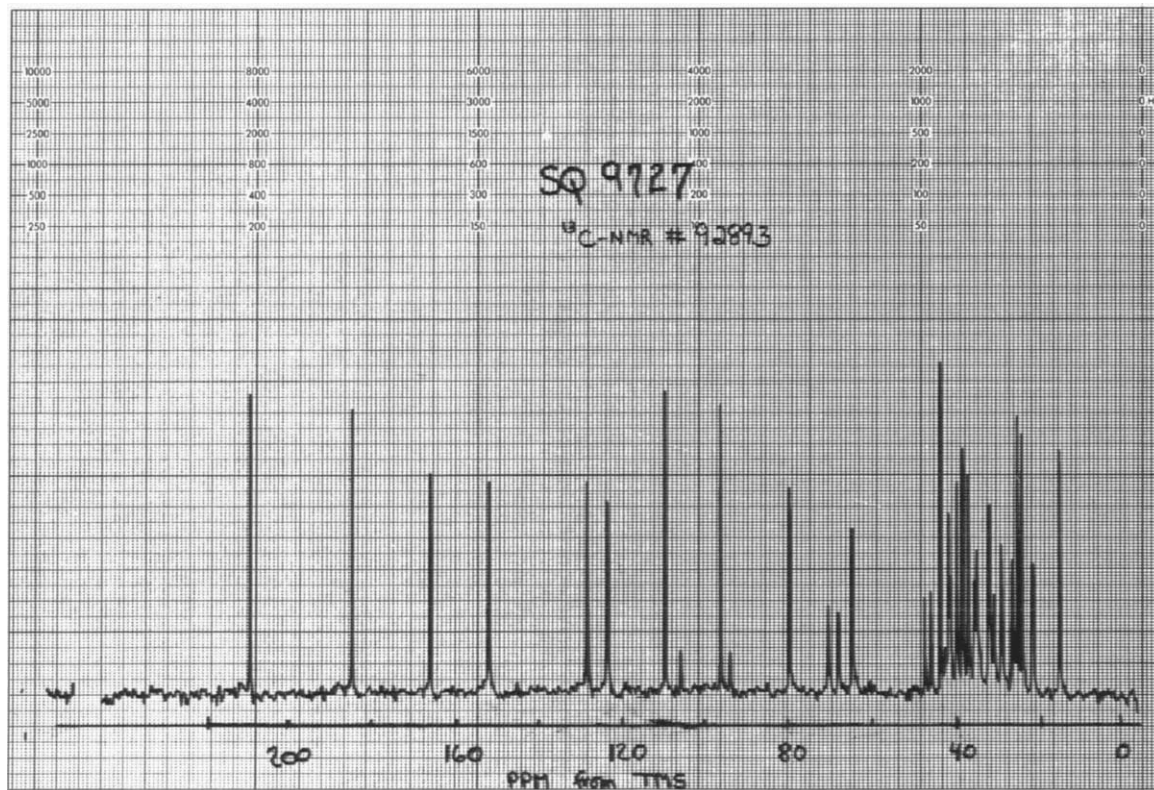


Figure 2. ^{13}C -Nuclear magnetic resonance spectrum of triamcinolone acetonide (SQ 9727) in dimethylsulfoxide- d_6 . (Instrument: JEOL FX60Q).

(dimethylsulfoxide - d_6 = 39.5 PPM) and ^{13}C - ^{19}F coupling constants and are consistent with literature values for closely related compounds⁵. The assignments were simplified by the utilization of polarization transfer to selectively enhance and phase alter individual carbon types⁶.

Table I. ^{13}C -NMR Spectral Assignments^a of Triamcinolone Acetonide

Carbon Number	Chemical Shift ^b	Carbon Number	Chemical Shift
1	152.4	13	44.7
2	129.0	14	42.8
3	185.1	15	33.0
4	124.2	16	80.8
5	166.5	17	97.2
6	30.1	18	16.3
7	27.5	19	22.7(5.8)
8	32.5(16.6) ^c	20	209.7
9	100.9(175.8)	21	65.9
10	47.3(22.4)	-C- ^d	110.4
11	70.4(36.1)	CH ₃ ^e	26.3
12	36.6		25.3

^a All chemical shifts are in PPM from tetramethylsilane with internal reference dimethylsulfoxide- d_6 = 39.5 PPM. ^b All spectra were determined in DMSO- d_6 . ^c ^{13}C - ^{19}F coupling constants shown in parentheses. ^d Methylene carbon of the acetonide. ^e Methyl groups of the acetonide.

2.4 Mass Spectra

The low resolution electron impact mass spectra of several fluorinated corticosteroidal 1,4-diene-3-ones have been determined by Lodge and Toft⁷. The mass spectral fragmentation pattern found for triamcinolone acetonide is in excellent agreement with Florey¹. In addition, the mass spectra of triamcinolone acetonide 21-t-butylacetate was determined for comparison purposes.

The gas chromatographic-mass spectral characteristics of silanized triamcinolone

acetonide were determined by Painter⁸. Under the reaction conditions employed (see section 6.55 for reaction conditions and gas chromatographic details), triamcinolone acetonide was initially silanized at the C-21 position (m/z 506) and much slower at the C-11 position (m/z 578). An intense fragment at m/z 447 [loss of $\text{COCH}_2\text{OSi}(\text{CH}_3)_3$] from the disilanized material (m/z 578) helped confirm the structure.

2.9 Solubility Data

2.91 Solubility

Solubilization of 19 steroid hormones, including triamcinolone, triamcinolone acetonide and triamcinolone diacetate, by polyoxyethylene lauryl ether was reported by Tomida⁹ who also concluded that this solubilization is directly dependent upon the lipophilicity of the steroids. Triamcinolone acetonide was found to have a solubility of 4.95×10^{-5} M in water¹⁰, 20 mg/g in acetone¹⁰, 0.012 mg/ml in water and 1.44 mg/ml in 40% ethanol¹¹.

Dissolution rates of triamcinolone acetonide in aqueous media were determined at 28°C via a rotating-disc method¹². Triamcinolone acetonide solubility in distilled water ranged from 21 mcg/ml at 28°C to 33.6 mcg/ml at 50°C. The dissolution rate constant at maximum agitation was 4.51×10^{-3} hr⁻¹ cm². Solubilities and dissolution rates were markedly lower in potassium chloride solution. The blue tetrazolium assay (section 6.31) was used to determine the triamcinolone acetonide. When the same laboratory¹³ used ¹⁴C-triamcinolone acetonide to determine solubilities, lower values were obtained. Triamcinolone acetonide solubility in distilled water ranged from 17.5 mcg/ml at 28°C to 26.5 mcg/ml at 50°C.

2.92 Partition Coefficients

The partition coefficients of triamcinolone acetonide in 3 systems have been determined by Tomida⁹. He determined that the aqueous-micellar, octanol-water and ether-water partition coefficients were 569, 205, and 14.6, respectively. The values of the octanol-water and

ether-water partition coefficients are in excellent agreement with those published by other investigators^{11,14,15}.

The role of crystal structure (as reflected by the melting point and the entropy of fusion) and of the activity coefficient (as reflected by the octanol-water partition coefficient) in controlling the aqueous solubility of either liquid or crystalline non-electrolytes, including triamcinolone, triamcinolone acetonide and triamcinolone diacetate, was discussed by Yalkowsky and Valyani¹⁴.

Fairbrother¹⁷ found that a partition system between acetonitrile and n-hexane gave quantitative separation of triamcinolone acetonide, found in the acetonitrile phase, from petrolatum. Structural relationships between a large group of steroids, including triamcinolone acetonide, and their ether-water partition coefficients were explored by Flynn¹⁵. Correlation with biological activity was also discussed.

The correlation of partitioning and percutaneous absorption of topically applied triamcinolone acetonide has been studied by Lien and Tong³⁷. Partition coefficients of triamcinolone acetonide on a diatomaceous earth column have been determined¹⁶ (see section 6.56).

2.10 Crystal Properties

Powder X-ray diffraction data¹⁰⁴ indicate that triamcinolone acetonide may exist in two polymorphic forms. For example, polymorphic form 1 exhibited a characteristic peak at 6.1 Å°, often split with a smaller peak at 5.95 Å°. Polymorphic form 2, on the other hand, showed a large 6.3 Å° peak but none at 5.95 or 6.1 Å°. Several other differences in the diffractograms were also noted. Infrared spectroscopy and differential scanning colorimetry were unable to differentiate between the two polymorphs.

Several parameters of grain size distribution and particle nature of triamcinolone acetonide crystal suspensions were determined by polarization, electron transmission and electron scanning microscopies¹⁸.

The sizes of drug particles in eye ointment suspensions, including triamcinolone acetonide preparations, were measured microscopically by List and Groenig¹⁹ using a membrane filtering technique.

Murata and co-workers²⁰ have reported the observation of crystal forms of triamcinolone acetonide, triamcinolone diacetate and other glucocorticoids in sterile aqueous suspension by electron microscopy.

3. Synthesis

Acetylation of 9 α -fluoroprednisolone-21-acetate followed by deacetylation of the triacetate using potassium acetate in dimethylformamide at 110-120°C for 4 hours under a nitrogen atmosphere gave 9 α -fluoro-pregna-1,4,16-triene-11 β ,21-diol-3,20-dione diacetate. The resulting diacetate was oxidized with aqueous potassium permanganate in acetone-formic acid for 3 seconds in a flow system at -5°C to give 15 α -hydroxyprednisolone in 90% yield. Ketalization with acetone followed by treatment with sodium hydroxide in methanol at 0°C for 3 hours in a nitrogen atmosphere gave triamcinolone acetonide^{21,22}.

Toth and co-workers²³ patented a process where triamcinolone acetonide 21-nitrate was treated with hydrogen fluoride in chloroform for 3 hours at 0°C to give triamcinolone acetonide in an 83% yield.²⁴

Reyba²⁴ has patented a general procedure for the synthesis of acetal fluoro derivatives of steroids. For example, 5 grams of triamcinolone in 8 ml of acetone was treated with 2 ml of perchloric acid and stored at 15-17°C with agitation for 2 hours to give 3.31 grams of triamcinolone acetonide.

Castelli and Ascheri²⁵ developed and patented a one-step transformation of 16 α ,17 α -dihydroxy-9,11-epoxy-3-oxo-pregn-1,4-diene to triamcinolone acetonide. In a typical synthesis, one equivalent of acetone was added to a 50% hydrogen fluoride solution cooled to -30°C followed by the addition of one equivalent of the epoxide.

Microbial transformation of 16 α ,17 α -dihydroxy-3-oxo-pregn-4-ene-16,17-acetonide to

triamcinolone acetonide was accomplished by incubation with *Arthrobacter simplex* for 12 hours at 28°C²⁶.

A procedure for the synthesis of 16,17-dihydroxy steroid acetal and ketal derivatives, including triamcinolone acetonide and triamcinolone diacetate, has been patented²⁷.

5. Drug Metabolism and Pharmacokinetics

5.1 Drug Metabolism

Since 6 β -hydroxylation was found to be the major metabolic pathway for triamcinolone in vitro and in vivo, this pathway was suggested for the metabolism of triamcinolone acetonide. Kupfer and Partridge²⁸ found that the post mitochondrial supernate of rat liver homogenate, supplemented with NADPH, hydroxylates triamcinolone acetonide with 6 β -hydroxytriamcinolone acetonide being the major metabolite. Inducers of the hepatic microsomal monooxygenase system, phenobarbital and 1-benzyl-2-thio-5,6-dihydrouracil, enhanced the 6 β -hydroxylation²⁹.

Yamashita²⁹ found that the metabolism of triamcinolone acetonide by rat liver slices or homogenates proceeded at rates inversely proportional to the antiinflammatory potency of the compound. The liver material metabolized triamcinolone acetonide to 6 β -hydroxytriamcinolone acetonide.

Since esterification of steroid alcohols is known to have a marked influence on their absorption and excretion, the metabolism of ¹⁴C-triamcinolone acetonide 21-phosphate in dogs, monkeys and rats was studied by Kripalani and co-workers³⁰. They found that the ¹⁴C-labelled steroid was completely hydrolyzed to triamcinolone acetonide following intramuscular or intravenous injection. The radioactivity was eliminated rapidly ($t_{1/2}$ = 1 to 2 hours) from plasma and tissues and the major route of excretion was via the bile. 6 β -Hydroxytriamcinolone acetonide was found to be the major metabolite in urine of all three species. Also, metabolites present in the urine as glucuronides and sulfates accounted for approximately 10-15% of the radioactivity.

Recently, Gordon and Morrison³¹ studied the metabolic fate of ¹⁴C-triamcinolone acetonide in

rabbits, dogs, monkeys and rats. In the dog, rat and monkey the major excretory route was the feces irrespective of the mode of administration. In the rabbit the excreted radioactivity was equally distributed between urine and feces. The metabolites were isolated by preparative TLC, located by autoradiography and eluted and analyzed by MS, IR, UV and NMR. The major metabolites of triamcinolone acetonide were identified as the C-21 carboxylic acids of triamcinolone acetonide and of 6 β -hydroxytriamcinolone acetonide and the previously identified 6 β -hydroxytriamcinolone acetonide. In addition, MS and UV data indicated the presence of 9 α -fluoro-11 β ,16 α ,17-trihydroxy-3,20-dioxo-1,4,6-pregnatrien-21-oic acid cyclic 16,17-acetal. They were unable to find conjugation as a major mechanism of excretion.^{32,33}

Zimmerman and Bowen^{32,33} studied the teratogenic effects of triamcinolone acetonide on mice by monitoring the incidence of cleft palate. Since practically all of the radioactivity found in the embryos and placentas was unmetabolized drug, it was concluded that the triamcinolone acetonide and not its metabolites was the teratogenic agent.

5.2 Pharmacokinetics and Bioavailability

Since the desired site of activity for topical corticosteroids is the dermis or the epidermis, percutaneous absorption of these materials has been extensively studied. Local changes in the skin and subcutaneous tissue caused by percutaneous absorption was the subject of an extensive review article by Keipert³⁴ and included discussions of triamcinolone and triamcinolone acetonide.

Kukita and co-workers³⁵ reported that triamcinolone acetonide was absorbed percutaneously mainly by the transfollicular route. Hartmann and Ude³⁶ studied the effect of triamcinolone acetonide treatment with occlusive foil by monitoring the plasma cortisol, urinary cortisol and urinary triamcinolone acetonide through quantitative thin layer chromatography. Plasma cortisol and urinary cortisol values were also used by Rasmussen³⁷ to study the percutaneous absorption of triamcinolone acetonide in a 0.1%

ointment. Serum cortisol values were measured by a radioallergosorbent test.

Bioavailability and activity of triamcinolone acetonide using a novel drug delivery system, the aerosol quick-break foam, was studied by Woodford and Barry³⁸. In another study, Woodford and Barry³⁹ compared the bioavailability and activity of 0.1% triamcinolone acetonide and 0.1% amcinonide preparations to a 0.025% fluocinoline acetonide gel using a vasoconstrictor assay. Vericat and co-workers⁴⁰ evaluated the bioavailability of triamcinolone acetonide following topical application as a suspension in oleoalcoholic cream, suspension in fat excipient or polyalcoholic solution by a vasoconstrictor assay. Greatest bioavailability was observed with the alcoholic solutions of the compound. A method using the vasoconstrictive properties of corticoids as indicated by thermal conductivity to measure the amount and speed of these compounds, including triamcinolone acetonide, penetrating the skin after topical application was described by Tronnier⁴¹. Elimination biokinetics of 0.01, 0.10 and 1.0% triamcinolone acetonide creams using a vasoconstrictor assay has been reported by Barry and Brace⁴².

Quantitative determination of percutaneous absorption of radiolabeled drugs, including ³H-triamcinolone acetonide, was described by Schaeffer⁴³ and co-workers in a series of articles⁴³⁻⁴⁷. Using autoradiographic techniques, a rapid penetration into the living layers of the skin was observed. However, total excretion in the urine took more than 72 hours after removal of the excess of substance from the skin. Autoradiographic techniques were also used to quantitate the percutaneous absorption of ³H-triamcinolone acetonide in human skin^{48,49}, animal skin^{48,49} and in human skin from lanolin alcohol-ethyl cellulose film^{52,53}. Penetration of triamcinolone acetonide through epidermal membranes was greatly enhanced by the addition of salicylic acid⁵⁴.

The influence of keratolytics and moisturizers on the bioavailability of triamcinolone acetonide using the blanching effect as the parameter of detection was examined by Gloor and Lindemann⁵⁶.

Verma and co-workers¹¹ compared the potency and effectiveness of fluorinated steroids like triamcinolone acetonide and nonfluorinated steroids such as 16 α -hydroxyprednisolone acetonide by studying solubility, partition coefficients, vasoconstrictor ability and penetration across human epidermis. They concluded that the 9 α -fluoro group has no effect on the potency of triamcinolone acetonide. The correlation of physicochemical properties i.e., lipophilicity as measured by partitioning in suitable solvent systems, molar refraction, Taft's polar substituent constant (σ°), molecular weight and water solubility, and percutaneous absorption of topically applied drugs, including triamcinolone acetonide, has been studied by Lien and Tong⁵⁷.

Biagi and co-workers⁵⁹ showed that the chromatographic R_m value of triamcinolone acetonide could be correlated with its lipophilic character. Therefore, the dependence of protein binding absorption and biotransformation on lipophilic character strongly influences the availability of steroids at the site of action.

Hackney and co-workers⁵⁸ demonstrated that mouse fibroblasts growing in vitro contain a binding component for triamcinolone acetonide which is apparently distributed intracellularly, largely as a cytoplasmic soluble macromolecule. Furthermore, the structure-activity relationships of steroids active in growth inhibition were found to be exactly paralleled by their ability to displace ³H-triamcinolone acetonide from this binding component.

A new, selective drug delivery system, which uses liposomes as drug carriers, for the topical route of administration⁵⁵ was recently described by Mezei and Gulasekharan⁵⁵. Using ¹⁴C-triamcinolone acetonide, they found that liposomal encapsulation increased the concentration of the drug at the site where its activity is desired i.e., epidermis and dermis. Because of the selectivity of the liposomal dosage form, it could provide increased efficacy and decreased toxicity of topically, applied drugs.

6. Methods of Analysis

6.2 Direct Ultraviolet Analysis

In the ultraviolet spectrum of a mixture of triamcinolone acetonide and chlorhexidine, the overlapping absorption maxima were approximated by the use of Gauss-Lorenz function parameters for calculations on a programmed computer⁶⁰. Polysorbate 80 and methyl- and propyl-p-hydroxybenzoates were found to interfere in the determination of corticosteroids such as triamcinolone acetonide in dermatological preparations by the ultraviolet spectrophotometric method⁶¹.

6.3 Colorimetric Analysis

6.31 Ochipinti and co-workers⁶² reported significant improvements in the tetrazolium blue method for the determination of steroids containing an α -ketol side chain. They reported optimal concentration and experimental conditions for rapid quantitative determinations of 17 representative steroids, including triamcinolone and triamcinolone acetonide, occurring in pharmaceutical formulations. Tsuchikura and co-workers⁶³ found that the 1,2-double bond, the 16 α -hydroxy and the 16,17-acetonide groups inhibited the blue tetrazolium reaction.

Notable interference was shown by polyethylene glycols, propylene glycol and lanolin in the determination of corticosteroids such as triamcinolone acetonide in dermatological preparations by the blue tetrazolium method⁶¹. Sorbitol and squalene caused only slight interference.

A reaction rate method using a modified blue tetrazolium reaction for the determination of triamcinolone acetonide has been described⁶⁴. This method depends on the mixing of a 5% tetramethylammonium hydroxide in absolute ethanol solution with a blue tetrazolium solution of triamcinolone acetonide by an automatic stopped-flow system and monitoring the absorbance at 525 nm during selected measurement time.

6.32 Tsuchikura and co-workers⁶³ found that the 1,2-double bond, the 16 α -hydroxy and the

16,17-acetonide groups inhibited the isonicotinic acid hydrazide reaction of steroids. Yamashita and co-workers⁶⁵ investigated the quantitative and qualitative determination of synthetic corticoids and reported the isonicotinic acid hydrazide determination of triamcinolone and triamcinolone acetonide. Substances including carbonyl, reducing, acidic and basic compounds interfered with the determination of triamcinolone acetonide in ointment preparations by the isonicotinic acid hydrazide method⁶⁶. Girard's fractionation method was used to remove the interfering substances prior to measurement.

6.33 The reaction of a 1 mg/ml aqueous solution of sulfuric acid with triamcinolone acetonide produced a faintly yellow color in daylight and no color when viewed with an ultraviolet lamp at 366 nm⁶⁷.

6.34 Although the Porter-Silber test (phenylhydrazine and sulfuric acid) has been used for the determination of triamcinolone acetonide⁶³, it is not recommended because of the low molar absorptivity⁶⁷ of the chromogen produced.

6.35 The reaction of triamcinolone acetonide with 2,3,5-triphenyltetrazolium chloride instead of tetrazolium blue followed by absorbance measurement according to the European Pharmacopeia was used to quantitate the steroid in pharmaceutical preparations⁶⁸.

6.36 A new method based on the oxidation of the C-17 side chain with cupric acetate, condensation of the resulting 20-keto-21-aldehyde with 4,5-dimethyl-o-phenylenediamine and spectrophotometric measurement of the quinoxaline derivative obtained has been described by Szepesi and Görög⁶⁹ for the determination of 21-hydroxycorticosteroids, including triamcinolone acetonide, in pharmaceutical preparations. This procedure was used for the analysis of bulk material (purification on a cation exchange column was required after the oxidation step) or ointment (no purification required).

Chafetz and Tsilifonis⁷⁰ also used a cupric acetate oxidation step to determine triamcinolone

acetone. The oxidation step was followed by chromogen formation with aqueous acidic phenylhydrazine and absorption measurement at 370 nm. This procedure is claimed to be twice as sensitive as Szepesi and Görögs⁶⁹.

Bundgaard⁷¹ quantitatively determined triamcinolone acetone by cupric acetate oxidation followed by condensation with 3-methyl-benzothiazol-2-one hydrazone in alkaline solution to form the highly absorbing azine ($\lambda_{\text{max}} = 400 \text{ nm}$). This method was used to determine triamcinolone acetone in the presence of its 21-acetate ester.

6.4 Polarographic Analysis

Differential pulse polarography was used to study the electroanalytical behavior of triamcinolone acetone⁷². The half-wave potentials vs Ag/AgCl electrode of triamcinolone acetone were determined as -1.46 V (C-3 keto reduction) and -1.70 V (C-3 keto) in Britton-Robinson buffer pH 10 (50% v/v in methanol), -1.60 V (C-3 keto), -1.79 V (C-3 keto) and -2.03 V (C-3 keto) in 0.03 M tetramethylammonium hydroxide in methanol and -1.71 V (C-3 keto), -1.98 V (C-3 keto) and -2.15 V (C-20 keto) in 0.02 M tetramethylammonium hydroxide in 87% aqueous dimethylformamide. This electroanalytical technique was then used to determine triamcinolone acetone in multicomponent and complex pharmaceutical preparations⁷³.

6.5 Chromatographic Analysis

6.52 Thin Layer Chromatographic Analysis

A compilation of the thin layer chromatographic properties and identification of triamcinolone acetone published after 1970 is shown in Table II.

Identification¹⁰³ of triamcinolone acetone in a 0.025% ointment¹⁰³ was accomplished by thin layer chromatography on silica gel GF plates using a developing solvent of methanol: chloroform: benzene (20:100:40) and a 0.2% blue tetrazolium in methanol spray reagent. Massa⁷⁵ identified triamcinolone acetone by thin layer chromatography on silica gel using a developing

solvent of benzene:methanol (85:15) and fluorescence at 366 nm.

6.54 High Performance Liquid Chromatography

Recently, a high-performance liquid chromatographic (HPLC) procedure involving the use of adsorption chromatography on Corasil II has⁸⁴ been adopted by the United States Pharmacopeia⁸⁴ for the determination of triamcinolone acetonide in 0.1 and 0.025% creams. A similar method⁸⁵ with a silica gel column and a solvent system of 95% ethanol:methylene chloride (5:95) has been used for the analysis of triamcinolone acetonide creams. Triamcinolone acetonide has also been determined in rice starch⁸⁶ using adsorption HPLC. These methods, which do not involve the use of an internal standard, and numerous other methods for the detection and determination of triamcinolone acetonide with internal standards are listed below in Table III.

Table II. Thin Layer Chromatography of Triamcinolone Acetonide

Solvent System	Adsorbent	Detection	R _f	Reference
Methylene Chloride: methanol (15:6)	Silica gel	Radioactive scanner	0.91	79
A-Methylene Chloride: methanol (93:7)				
B-Cyclohexane: dioxane:water (50:50:10)				
Equal volumes of A and upper phase of B			0.36	
Benzene:acetone (2:1)			0.22	
Equal volumes of A and upper phase of B:methanol (9:2)			0.64	
Methanol:ethyl (2:98)	Silica gel H	Radioactive scanner	0.45	30

Table II. Thin Layer Chromatography of Triamcinolone Acetonide (Continued)

Solvent System	Adsorbent	Detection	R _f	Reference
Toluene:ethyl- acetate:85% formic acid (50:45:5)	Kieselgel F-254	UV at 254 nm	0.38	78
Toluene:isopropanol: 37% ammonium hydroxide (70:29:1)			0.59	
Toluene:dioxane: methanol:37% ammonium hydroxide (20:50:20:10)			0.84	
Methylene chloride: diethylether: methanol:water (77:15:8:1.2)	Silica gel GF 254	UV at 254 nm	0.45	79
1,1-dichloroethanol: acetone:acetic acid (160:40:10)			0.42	

Table II. Thin Layer Chromatography of Triamcinolone Acetonide (Continued)

Solvent System	Adsorbent	Detection	R _f	Reference
Toluene:chloroform (75:25) Stationary phase- Formamide:acetone (10:90)			0.43	
Ethylene dichloride: methanol:water (95:5:0.2)			0.13	
Ethylene chloride: methanol:water (95:5:0.2)	Silica gel GF 254	50% ethanolic sulfuric acid, 120°C for 5 minutes	0.65 relative to 16β- methylpredni- solone acetate = 1.00	80
Butylacetate or Dichloroethane: methyl acetate: water (2:1:1), lower layer Stationary phase - dioxane	Kieselgel 60 F 254	Spray with 20% anhydrous zinc chloride in methanol, 110°C for 20 minutes, UV at 254 and 366 nm	Identity Test	81

Table II. Thin Layer Chromatography of Triamcinolone Acetonide (Continued)

Solvent System	Adsorbent	Detection	R _f	Reference
Chloroform: methanol:glacial acetic acid (90:10:2)	Silica gel	UV at 254 nm	0.47	82
Toluene:chloroform (75:25) Stationary phase-Formamide: acetone (10:90)	Kieselguhr G	Spray with 1 mg/ml sulfuric acid, violet color at 366 nm	0.39	67
Methylene chloride: diethylether: methanol:water (77:15:8:1.2)	Silica gel G		0.13-0.15	
Chloroform: methanol:acetic acid (90:10:2)	Silica gel	Fluorescence at 2537A°	0.47	83

Table III. High Performance Liquid Chromatography of Triamcinolone Acetonide

Column	Mobile Phase	Flow Rate (ml/min)	Retention Time (min)	Detection (nm)	Reference
16 reverse phase ODS, RP and phenyl columns	Acetonitrile: water (30:70)	2.0	Variable	254	87
Hypersil ODS 20 cm	Methanol: water (60:40)	1.0		250	88
10 μ silica Gel 25 cm x 0.2 cm	95% ethanol: methylene chloride (5:95)	1.55	1.28	254	85
μ Bondapak C ₁₈ 30 cm x 4.0 mm	Methanol:water (70:30)	1.5	1.50	254	89
	Methanol:water (50:50)		4.93		
	Acetonitrile: water (60:40)		1.22		
	Acetonitrile: water (40:60)		2.21		
			Relative to acetone 1.00		

Table III. High Performance Liquid Chromatography of Triamcinolone Acetonide (Continued)

Column	Mobile Phase	Flow Rate (ml/min)	Retention Time (min)	Detection (nm)	Reference
Bondapak C ₁₈ / Corasil 6l cm x 2.3 mm	Methanol:water (60:40)	1.5	1.22		
	Methanol:water (40:60)		6.41		
	Acetonitrile: water (40:60)		1.11		
	Acetonitrile: water (20:80)		8.12		
			Relative to acetone 1.00		
Bondapak Phenyl/Corasil	Methanol:water (60:40)	1.0	1.28		
	Methanol:water (40:60)		5.40		
	Acetonitrile: water (40:60)		1.17		
	Acetonitrile: water (20:80)		4.56		
			Relative to acetone 1.00		
Zipax 1 m x 2 mm	water- saturated methylene chloride	0.33	7.2	254	86

Table III. High Performance Liquid Chromatography of Triamcinolone Acetonide (Continued)

Column	Mobile Phase	Flow Rate (ml/min)	Retention Time (min)	Detection (nm)	Reference
Spherisorb ODS 5-10 25 cm x 4.6 mm	Methanol:water (56:44)	1.3-1.5	11.0	254	90,91
Biosil A 20-44 μ 50 cm x 2.1 mm	Petroleum ether:chloro- form:methanol (60:39:2)	1.0 or 250 kg/cm ²	19.0	254	92
	Methylene chloride:meth- anol:water (97:1:2)		26.6		
	Methylene chloride: isopropanol (98:2)		13.3		

Table III. High Performance Liquid Chromatography of Triamcinolone Acetonide (Continued)

Column	Mobile Phase	Flow Rate (ml/min)	Retention Time (min)	Detection (nm)	Reference
10% ODS μ - Bondapak	Acetonitrile: water (30:70)	2.0	12.0	254	93
5% ODS Partisil			6.0		
3-Reversed Phase ODS columns	Acetonitrile: water (60:40)	2.0	6.0	254	94

Quantitative determination of triamcinolone acetonide in a 0.0152% aerosol spray by HPLC has been reported by Gibbs and Kirschbaum⁹⁵. Using halcinonide as the internal standard, separation and quantitation was accomplished on a reverse phase Partisil ODS 25 cm x 4.6 mm column with a mobile phase of acetonitrile:water (40:60), a flow rate of 1.0 to 1.5 ml/min and UV detection at 254 nm.

A procedure has been developed by Tenneson⁸⁸ for the determination of triamcinolone acetonide in human serum by HPLC with a detection limit of 1 ng/ml. The triamcinolone acetonide is extracted from potassium chloride saturated serum samples into diethylether. After evaporation, the residue is taken up in the mobile phase (methanol:water, 60:40) and then separated (retention time 10 minutes) and quantified on a 5 micron Hypersil ODS HPLC column monitoring the absorbance of the eluate at 250 nm.

6.55 Gas Liquid Chromatography

Three gas liquid chromatographic (GLC) systems for the separation and identification of triamcinolone acetonide are shown below in Table IV. In addition to those listed below, the gas chromatographic properties of triamcinolone acetonide that had been silanized at C-11 and disilanized at C-11 and C-21 have been studied by Painter⁸. Triamcinolone acetonide was silanized by reaction with trimethylsilylimidazole:bis-trimethylsilyl acetamide:trimethylchlorosilane (3:3:2) and chromatographed on a 5 ft. x 0.020 in OV-1 stainless steel support coated open tubular column held at 220°C. The direct injector and flame ionization detector temperatures were 220 and 300°C, respectively. The helium carrier gas pressure was 5.0 psig. The disilanized product eluted at 2.5 minutes. The mass spectral characteristics of the silanized products were also determined (section 2.4).

Table IV. GLC Systems for the Separation and Identification of Triamcinolone Acetonide

Column	Column T (°C)	Detection, carrier gas, flow rate	Derivati- zation	R _f	Reference
1% OV-17 on 100/120 silanized Supasorb 9 ft. x 0.25 in	260	EC, nitrogen, 100 cc/min	tri- methyl- silyl	31.5 min	96
3% OV-17 on Chromosorb WHP 1.8 m x 2.0 mm	260	FID, helium, 60 cc/min	tri- methyl- silyl	4.38 min	97
3% OV-3 on Chromosorb G	250	EC, nitrogen, 60 cc/min	tri- methyl- silyl	16 min	98

6.56 Column Chromatography

The versatile solvent system of n-hexane:-chloroform:dioxane:water (90:10:40:5) was used for the partition chromatographic isolation of triamcinolone acetonide¹⁶. The partition coefficients were determined on a diatomaceous earth column.

6.6 Fluorimetric Analysis

Kadin⁷⁴ has described a fluorimetric determination of corticosteroids containing the $\Delta^{1,4}$ -3-keto group. These A-ring dienone-containing steroids were reacted with zinc dust and 40% sulfuric acid in n-butyl ether and the fluorescence emission and activation maxima determined at 390 and 340 nm, respectively. This simple method allowed the quantitation of triamcinolone acetonide in liquid formulations with a sensitivity of about 2 micrograms. Under the conditions employed only steroidal A-ring dienones fluoresce.

Fluorescent silica gel chromatography and photodensitometry have been used to determine triamcinolone acetonide in pharmaceutical formulations⁷⁵.

6.7 Titrimetric Analysis

Steroids with a 16 α ,17 α -diol group can be titrated with lead tetraacetate in acetic acid to a potentiometric endpoint. The micro version of this method using a 0.001 N lead tetraacetate solution enabled triamcinolone contaminant to be determined in triamcinolone acetonide^{76,77}.

6.8 Differential Borohydride Analysis

The differential borohydride assay developed by Görög¹⁰⁰ is an excellent method for the determination of Δ^4 - and $\Delta^{1,4}$ -3-ketosteroids in pharmaceutical preparations. Addition of propylene glycol to the reaction mixture to complex sodium metaborate allowed Kirschbaum¹⁰² to improve the reduction of Δ^4 - and $\Delta^{4,6}$ -3-ketosteroids. However, $\Delta^{1,4}$ -3-ketosteroids i.e., triamcinolone were less than 10% reduced. By the use of lithium borohydride instead of sodium borohydride, Chafetz and co-workers¹⁰¹ were able to determine

triamcinolone. This method should also allow the determination of triamcinolone acetonide.

6.9 Radioimmunoassay

A radioimmunoassay for triamcinolone acetonide in plasma was described by Ponec and co-workers⁹⁹. The antibody against triamcinolone acetonide was obtained by immunizing rabbits with triamcinolone acetonide-21-hemisuccinate coupled to bovine serum albumin. The minimum detectable amount was 200 pg.

7. Determination in Body Fluids and Tissues

Triamcinolone acetonide has been determined in biological fluids and tissues by several different methods of analysis in combination with various extraction techniques. Table V summarizes the results that have been published since 1970.

Table V. Determination of Triamcinolone Acetonide in Biological Fluids and Tissues

Chromatographic Method	Extraction	Biological System	Internal Standard	Reference
GLC	Ethyl acetate followed by partitioning between hexane and 70% aqueous methanol	Rat muscle	³ H-triamcino-lone acetonide	96
GLC	Ethyl acetate	Mouse and human dermal fibroblasts	Progesterone	97
TLC with fluorescence	Dichloro-methane	Plasma and urine	³ H-triamcino-lone acetonide	82,83
HPLC	Diethylether	Human serum		88

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TRIAMCINOLONE DIACETATE

David H. Sieh

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The following supplement contains updated information pertaining to the analytical chemistry of triamcinolone diacetate. A literature survey was conducted and is complete up to July 1981. The numbering system for topics discussed is the same as that in the original profile (Volume 1, pp. 422-442).

1. Description

Triamcinolone diacetate is a glucocorticoid used primarily in the treatment of adrenocortical and rheumatic disorders.

1.13 The CA Registry Number for triamcinolone diacetate is 67-78-7.

2. Physical Properties

2.2 Nuclear Magnetic Resonance

The improved ^1H -NMR and the ^{13}C -NMR spectra² of triamcinolone diacetate are shown in Figures 1 and 2, respectively (please refer to Figure 3 section 2.2 of the original profile¹ for comparison and assignments of the ^1H -NMR spectrum). The ^{13}C -spectrum was determined on a JEOL FX60Q spectrometer using a 10 mm C/H dual probe. A complete assignment for all the carbon atoms in the ^{13}C -NMR spectrum is listed in Table I. The assignments are based on relative chemical shifts (dimethylsulfoxide- d_6 = 39.5 PPM) and ^{13}C - ^{19}F coupling constants and are consistent with literature values for closely related compounds³. The assignments were simplified by the utilization of polarization transfer to selectively enhance and phase alter individual carbon types⁴.

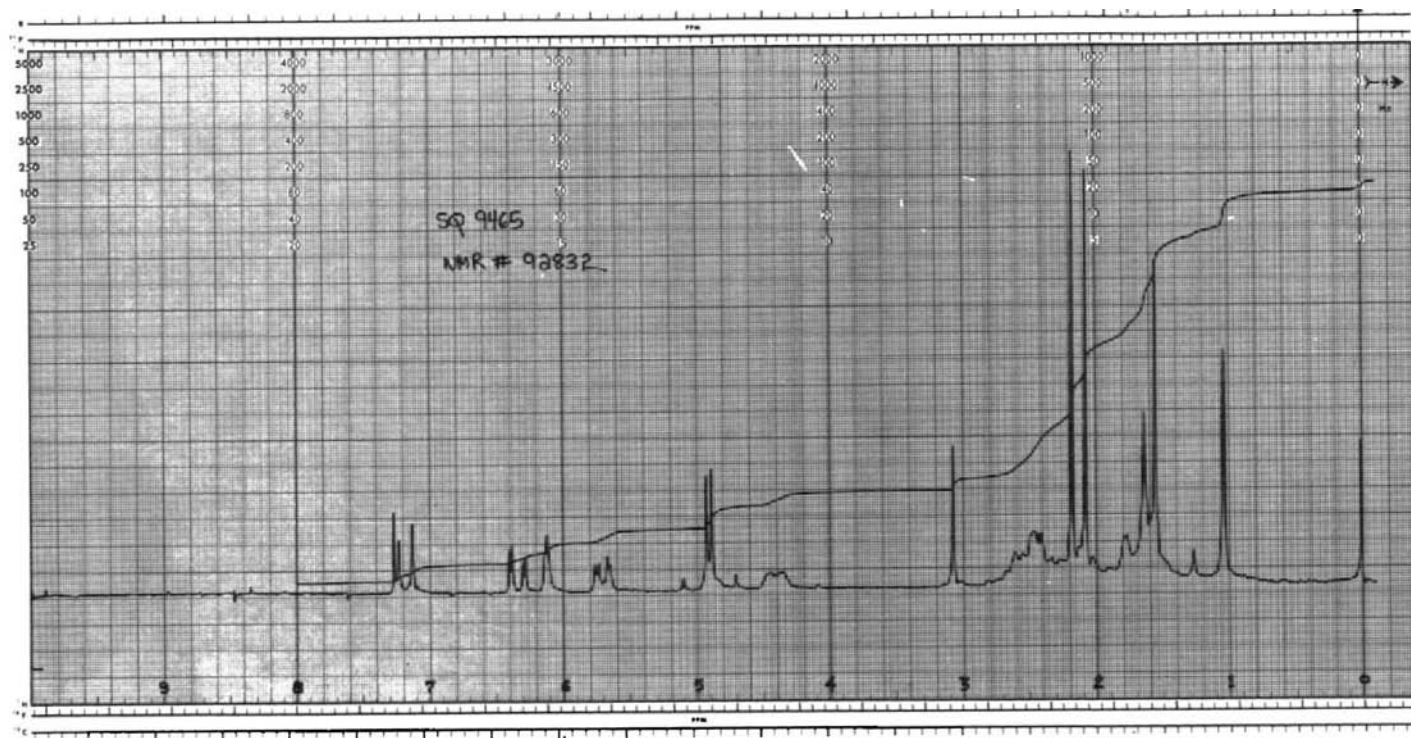


Figure 1. ^1H -Nuclear magnetic resonance spectrum of triamcinolone 16,21-diacetate (SQ 9465) in CDCl_3 . Instrument: Varian XL-100A.

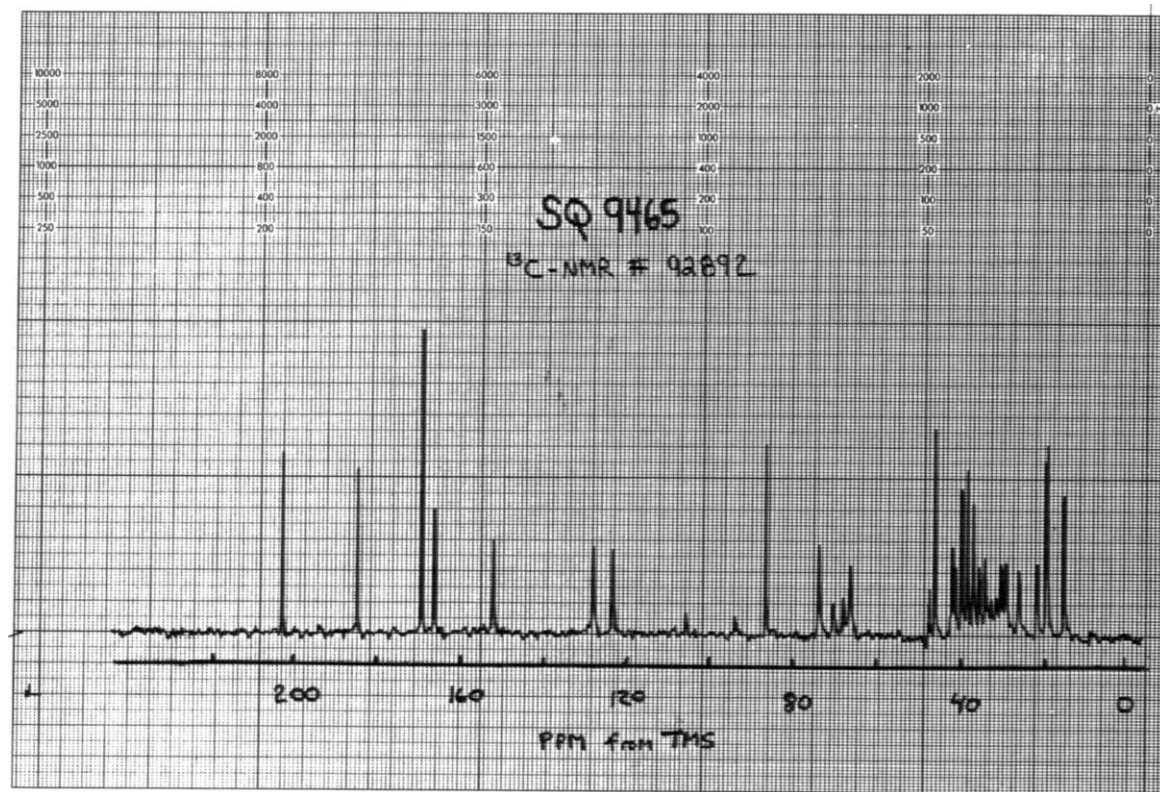


Figure 2. ^{13}C -Nuclear magnetic resonance spectrum of triamcinolone 16,21-diacetate (SQ 9465) in dimethylsulfoxide- d_6 . Instrument: JEOL FX60Q.

Table I. ^{13}C -NMR Spectral Assignments^a of Triamcinolone 16,21-diacetate

Carbon Number	Chemical Shift ^b	Carbon Number	Chemical Shift
1	152.7	13	47.4
2	129.0	14	43.1
3	185.3	15	31.6
4	124.2	16	75.1
5	166.9	17	87.8
6	30.3	18	16.3
7	27.2	19	23.0(4.9)
8	33.2(18.5) ^c	20	203.4
9	101.0(175.8)	21	67.5
10	48.0(20.5)	C=O	169.8
11	70.4(36.1)	CH ₃	20.8
12	35.3		20.3

^aAll chemical shifts are in PPM from tetramethylsilane with internal reference dimethylsulfoxide- d_6 = 39.5 PPM. ^bAll spectra were run in DMSO- d_6 . ^c ^{13}C - ^{19}F coupling constants shown in parentheses.

2.4 Mass Spectra

The low resolution mass spectra of 28 corticosteroid 21-esters and related compounds of pharmaceutical interest have been determined by Toft and co-workers⁵ on an AEI MS-12 via direct probe. The mass spectra of triamcinolone and triamcinolone diacetate are in excellent agreement with Florey¹.

2.9 Solubility Data

2.91 Solubility

Solubilization of 19 steroid hormones, including triamcinolone, triamcinolone acetonide and triamcinolone diacetate by polyoxyethylene lauryl ether has been reported by Tomida and co-workers⁶ who also concluded that the solubilization of steroids by polyoxyethylene lauryl ether micelles was directly dependent upon their lipophilicity. Triamcinolone diacetate was found

to have a solubility of 7.417×10^{-5} M in water⁶ and 13.2 mg/g in 70% ethanol⁷.

2.92 Partition Coefficients

The role of crystal structure (as reflected by the melting point and the entropy of fusion) and of the activity coefficient (as reflected by the octanol-water partition coefficient) in controlling the aqueous solubility of either liquid or crystalline organic nonelectrolytes, including triamcinolone, triamcinolone acetate and triamcinolone diacetate was discussed by Yalkowsky and Valvani⁸.

The partition coefficients of triamcinolone diacetate in two systems have been reported by Tomida⁹. He determined that the aqueous-micellar and octanol-water partition coefficients were 299 and 83.7, respectively. The value for the octanol-water partition coefficient is in excellent agreement with that published by other investigators⁸⁻¹⁰.

2.10 Crystal Properties

Murata and co-workers have reported the observation of crystal forms of triamcinolone diacetate and other glucocorticoids in sterile aqueous suspensions by scanning electron microscopy.

3. Synthesis

Higashikawa¹² has patented a procedure for the preparation of triamcinolone diacetate by reacting the 21-hydroxy precursor with acetic anhydride in the presence of metal carbonate or hydroxide. A procedure for the synthesis of 16,17-dihydroxy steroid acetal and ketal derivatives and acetylated compounds has been patented¹³.

6. Methods of Analysis

6.3 Colorimetric Analysis

6.34 The reaction of a 1 mg/ml aqueous solution of sulfuric acid with triamcinolone diacetate produced a very faint color in daylight

and no color when viewed in the ultraviolet at 366 nm¹⁴.

6.37 Triamcinolone diacetate has been quantitated by its reaction with 2,3,5-triphenyl-tetrazolium chloride followed by absorbance measurement¹⁵.

6.5 Chromatographic Analysis

6.52 Thin Layer Chromatographic Analysis

Four systems used for the separation and identification of triamcinolone diacetate by thin layer chromatography are shown in Table II.

6.54 High Performance Liquid Chromatography

A compilation of the liquid chromatographic separation systems for triamcinolone diacetate published after 1970 is shown in Table III. K' is defined as the capacity ratio.

7. Acknowledgements

The author would like to express his appreciation to Mr. Steve Highcock for performing the literature search, to Dr. Mike Porubcan for determining and analyzing the nuclear magnetic resonance spectra, and to Dr. John Dunham for his critical review of the manuscript.

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TABLE II. Thin Layer Chromatography of Triamcinolone Diacetate

Solvent System	Adsorbent	Detection	R _f	Reference
Methylene chloride: dioxane: water (10:5:5)- lower layer	Silica gel GF 254	Spray with 50% sulfuric acid, heat at 120°C for 5 min.	0.88	16
Ethylene chloride: methanol: water (95:5:0.2)			1.00 Relative to 16β- methyl predniso- lone ace- tate-1.00	
Chloroform Stationary phase - Formamide: acetone (10:90)	Kieselguhr G	Spray with 1 mg/ml sul- furic acid, uv at 366 nm	0.18	14
Methylene chloride: diethylether:methanol: water (77:15:8:1.2)	Silica Gel G		0.27	

Table III. High Performance Liquid Chromatographic Systems for Triamcinolone Diacetate.

Column	Mobile Phase	Flow Rate (ml/min)	Retention Time (min)	Detection (nm)	Reference
Zipax 1m x 2mm	Water saturated methylene chloride	0.33	4.8	254	17
μ Bondapak C ₁₈ 30cm x 3.9 mm	Acetonitrile: water (30:70)	1.0	K'=8.48	240	18
μ Bondapak C ₁₈ 30 cm x 4.0mm	Methanol:water (70:30) Methanol:water (50:50) Acetonitrile:water (60:40) Acetonitrile:water (40:60)	1.5	1.30 3.38 1.25 2.65 Relative to acetone 1.00	254	19
Bondapak C ₁₈ / Corasil 61 cm x 2.3 mm	Methanol:water (60:40) Methanol:water (40:60) Acetonitrile:water (40:60) Acetonitrile:water (20:80)		1.33 4.88 1.11 9.06 Acetone = 1.00		

Table III. High Performance Liquid Chromatographic Systems for Triamcinolone Diacetate.

Column	Mobile Phase	Flow Rate (ml/min)	Retention Time (min)	Detection (nm)	Reference
Bonda- pak- Phenyl/ Corasil 6l cm x 2.3 mm	Methanol:water (60:40)	1.0	1.21		
	Methanol:water (40:60)		5.60		
	Acetonitrile:water (40:60)		1.21		
	Acetonitrile:water (20:80)		6.31		
			Acetone = 1.00		

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